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**UTILITY APPLICATION FOR LETTERS PATENT**

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**TO ALL WHOM IT MAY CONCERN:**

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Be it known that we, Christopher T. Ritchlin, Sally Haas-Smith, and Edward Schwarz, residing respectively at 4459 Middle Cheshire Road, Canadagua, New York 14424, 6 Craigmoores Road South, Ridgefield, Connecticut 06877, and 125 Barclay Square Drive, Rochester, New York 14618 have invented new and useful improvements in

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**METHODS AND COMPOSITIONS RELATED TO  
JOINT INFLAMMATION DISEASES**

**for which the following is a specification.**

## **METHODS AND COMPOSITIONS RELATED TO JOINT INFLAMMATION DISEASES**

1. This application claims benefit of U.S. Provisional Application No. 60/454,573, filed March 14, 2003, which is incorporated herein in its entirety.

2. This work was funded by NIAMS #AR 47186-01 and a Howard Hughes Pilot Grant. This work was also funded by National Institutes of Health grants PHS AR45791, AR43510, AR44220, and AR48697. The United States Government may have certain rights in the inventions disclosed herein.

### **I. BACKGROUND**

3. Inflammatory joint diseases, such as psoriatic and rheumatoid arthritis, involve a number of debilitating symptoms for those who suffer from them. The underlying reasons for these symptoms include bone and cartilage degradation. This degradation occurs through bone resorption, which is a process wherein osteoclast cells degrade existing bone. The present application discloses that the increase in osteoclast cells is preceded by an increase of osteoclast precursor cells (OPCs) in the peripheral blood of a subject with an inflammatory joint disease. Disclosed are methods of diagnosing inflammatory joint disease, as well as treatments for inflammatory disease, among other methods and compositions related to osteoclasts and osteoclast precursor cells and inflammatory joint diseases.

### **II. SUMMARY**

4. Disclosed are methods and compositions related to inflammatory joint disease.

### **III. BRIEF DESCRIPTION OF THE DRAWINGS**

5. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments and together with the description illustrate the disclosed compositions and methods.

6. Figure 1 shows large numbers of osteoclasts arise from unstimulated PsA PBMC. PBMC were obtained from 24 PsA patients and 12 healthy controls, cultured in the absence of M-CSF and RANKL for 14 days, fixed and stained for TRAP. The number of TRAP+ multinucleated cells (osteoclasts) were counted and are presented as osteoclasts per million PBMC plated.

7. Figure 2 shows culture PsA PBMC erode bone in a wafer assay. PBMC were cultured with or without M-CSF and RANKL on cortical bovine bone wafers. After 21 days the wafers were stained with toluidine blue to identify resorption lacunae. The percentages of eroded surface area on the wafers from unstimulated cultures from PsA (n=6) and healthy controls (n=6) were quantified as described in Methods.

8. Figure 3 shows OCP frequency in peripheral blood is greater in PsA patients with erosive arthritis. PBMC were obtained from 10 PsA patients with and 10 PsA patients without erosions on plain radiographs. The cells were cultured, fixed, stained for TRAP, and osteoclast numbers were determined as described in Methods. Data expressed as median OCP per  $10^6$  PBMC.

9. Figure 4 shows Anti-TNF- $\alpha$  therapy reduces OCP frequency in patients with PsA. (A) PBMC from five PsA patients were cultured to determine the OCP frequency before and following 12 weeks of anti-TNF- $\alpha$  therapy (4 with Enbrel® (etanercept) and one with infliximab). The data are expressed as OCP per  $10^6$  PBMC. The number of OCP in peripheral blood was significantly reduced in all of the patients ( $p < 0.001$ ). (B). The percentage of CD11b + PBMC significantly declined in 4 patients with erosive PsA, following 2 weeks of Enbrel® (etanercept) therapy as determined by FACS ( $p < 0.026$ ). Representative histograms of CD14/CD11b staining from a PsA patient before (C) and after Enbrel® (etanercept) therapy (D) are shown.

10. Figure 5 shows TNF- $\alpha$  produced by PsA PBMC enhances osteoclastogenesis. (A) Supernatants from cultured PsA PBMC were added to PBMC from healthy donors to stimulate osteoclastogenesis as described in the Examples. The mean number of osteoclasts that arose from 3 unstimulated healthy control PBMC cultures (open bar), incubated with MCSF and RANKL (gray bar), and co-cultured with PsA PBMC supernatants (black and striped bars) are presented as the mean  $\pm$  S.D. ( $*p < .02$ ). (B) To determine the effect of TNF- $\alpha$  in the co-cultures, PsA PBMC supernatant #2 from (A) was added to 2 different healthy control PBMC cultures and osteoclasts were counted with or without anti-TNF antibodies.

11. Figure 6 shows OPG inhibits osteoclast formation in unstimulated PsA PBMC. TRAP osteoclastogenesis assays were performed on PsA PBMC cultured without RANKL or M-CSF in the continuous presence of OPG-Fc (1  $\mu$ g/ml). Enbrel®

(etanercept) (1  $\mu$  g/ml) was added as indicated. One representative experiment out of 3 is shown. Data are expressed as the mean  $\pm$  SEM of four independent wells (\* $p$ <.05).

12. Figure 7 shows a schematic model of osteolysis in the psoriatic joint. Extensive erosions observed in the PsA joint are mediated by a bi-directional attack on bone. OCP can enter the synovium and are induced to become osteoclasts by RANKL expressed by synoviocytes (outside-in). In parallel, OCP traverse endothelial cells in the subchondral bone and undergo osteoclastogenesis following RANKL stimulation from osteoblasts/stromal cells (inside-out).

13. Figure 8 shows chronic systemic TNF $\alpha$  increases the osteoclast precursor frequency in spleen and blood. Splenocytes, blood cells, and adherent bone marrow cells from wild type (WT) or TNF-Tg mice were cultured with M-CSF and RANKL to determine their osteoclastogenic potential by TRAP assay (A, B); or with M-CSF in methylcellulose to determine their CFU-M colony-forming potential (C), as described in the Examples. The data are presented as the mean  $\pm$  SEM of 4 replicate samples (\* $p$ <0.005) from a representative mouse. Similar results were obtained with 2 additional pairs of wt and TNF-Tg mice.

14. Figure 9 shows that the CD11b<sup>+</sup> population is markedly increased in the spleen of TNF-Tg mice. Splenocytes from WT or TNF-Tg mice were stained with fluorescent probes specific for c-Kit, c-Fms, CD11b, or RANK and analyzed by FACS, as described in the Examples. The data are presented as the percentage of cells that were positive for the indicated surface protein from representative animals. A total of 5 pairs of WT and TNF-Tg mice were examined with similar results.

15. Figure 10 shows that all of the splenocytes with osteoclastogenic potential are in the CD11b<sup>hi</sup> population. Splenocytes from WT and TNF-Tg mice were stained with fluorescent antibodies specific for CD11b (A). The CD11b<sup>hi</sup> [R1], CD11b<sup>lo</sup> [R2], and CD11b<sup>-</sup> [R3] populations were sorted and cultured with M-CSF and RANKL, and their osteoclastogenic potential was determined by TRAP assay (B). The photographs show TRAP<sup>+</sup> osteoclasts only formed from CD11b<sup>hi</sup> [R1] cells. The data are representative of three independent experiments. Statistical analysis of the distribution of CD11b<sup>hi</sup> splenocytes from 16 pairs of mice shows the significant increase in the TNF-Tg vs. control (C).



16. Figure 11 shows the phenotypic characterization of the CD11b<sup>hi</sup> splenocyte population. Splenocytes from TNF-Tg mice were double-stained with anti-CD11b antibody in combination with probes for CD3, B220, F4/80, Gr-1, c-Kit, c-Fms, and RANK. Live CD11b<sup>hi</sup> cells were gated, and the histograms show the expression of the indicated markers in this population. The histograms are representative of three independent experiments.

17. Figure 12 shows that both CD11b<sup>hi</sup>/c-Fms<sup>-</sup> and CD11b<sup>hi</sup>/c-Fms<sup>+</sup> splenocytes form osteoclasts. Splenocytes from TNF-Tg mice were double-stained with antibodies for CD11b and c-Fms. CD11b<sup>hi</sup>/c-Fms<sup>-</sup> and CD11b<sup>hi</sup>/c-Fms<sup>+</sup> populations were sorted (A) and cultured as in Fig. 10, and the number of TRAP<sup>+</sup> osteoclasts was counted (B). The data are representative of two independent experiments.

18. Figure 13 shows the CD11b<sup>hi</sup> OCP frequency and serum human TNF $\alpha$  concentrations increase in TNF-Tg mice at 2-3 months of age. Splenocytes and blood were collected from TNF-Tg mice and their wt littermates at 1, 2, 3, and 4 months of age. The frequency of CD11b<sup>hi</sup> OCP in the spleen was determined by FACS (A). The concentration of human TNF $\alpha$  in the serum was analyzed by ELISA (B). The data from FACS are shown as representative animals (n  $\geq$  3 per group). No statistical differences were found in the frequency of CD11b<sup>hi</sup> OCP and the serum levels of human TNF $\alpha$  between 2, 3, and 4 months old TNF-Tg mice.

21. Figure 14 shows that the increased CD11b<sup>hi</sup> osteoclast precursor frequency in TNF-Tg mice is reversible with anti-TNF therapy. TNF-Tg mice were treated with peritoneal injections of Enbrel® (etanercept) (10 mg/kg) or placebo twice a week for 2 weeks. Splenocytes were isolated from these mice and analyzed for CD11b expression by FACS (A), osteoclastogenic potential by TRAP (B), and CFU-M forming potential by colony assay (C). The data are shown as representative animals (A) or the mean  $\pm$  SEM of 4 replicate samples (\*p<0.005 vs. WT, \*\*p<0.01 vs. PBS) from a representative mouse (B and C)(n = 3 per group). Similar results were obtained with 2 additional pairs of wt and TNF-Tg mice.

22. Figure 15 shows Systemic TNF $\alpha$  does not effect proliferation of CD11b<sup>hi</sup> in vivo. TNF-Tg and wt mice were labeled with BrdU for 24 hr as described in Methods. Splenocytes were isolated, double stained with antibodies for CD11b and BrdU, and analyzed by FACS. The percentage of CD11b<sup>hi</sup>/BrdU<sup>+</sup> cells is shown in dot plot (A).

Live CD11b<sup>hi</sup> cells were gated and the histograms showed the percentage of BrdU<sup>+</sup> cells in this population (B). Statistical analysis of 10 pairs of mice demonstrates no significant difference in the percentage of BrdU<sup>+</sup> cells in the CD11b<sup>hi</sup> population of TNF-Tg and wt mice (C).

5           23. Figure 16 shows that TNF $\alpha$  does not induce the differentiation of CD11b<sup>-lo</sup> to CD11b<sup>hi</sup> splenocytes *in vitro*. Wild type spleen cells were treated with TNF $\alpha$  (10 ng/ml) for 24 hr and subjected to FACS analysis using anti-CD11b antibody, and the percentage of CD11b<sup>hi</sup> cells is shown (A). Wild type total (B), sorted CD11b<sup>-</sup> and CD11b<sup>lo</sup> (C) splenocytes were treated with TNF $\alpha$  (10 ng/ml), and total RNA was  
10           extracted at various time points and analyzed by real-time PCR using CD11b specific primers. The data are representative of two independent experiments.

          24. Figure 17 shows that TNF $\alpha$  stimulates the release of CD11b<sup>+</sup> osteoclast precursors from the bone marrow to the periphery. (A-B) Eight-week-old wt mice were labeled with BrdU *in vivo* for 3 days and then challenged with TNF $\alpha$  (1  $\mu$ g/mouse, i.p)  
15           for 4 hr. Bone marrow cells (A) and blood cells (B) were collected, stained with antibodies for BrdU and CD11b, and analyzed by FACS. The percentage of CD11b<sup>+</sup>/BrdU<sup>+</sup> cells is shown on the upper-right corners, and that of CD11b<sup>+</sup>/BrdU<sup>-</sup> cells is in the upper-left corners. (C-D) Wild type mice were given intraperitoneal injections of PBS or TNF $\alpha$  (1  $\mu$ g/mouse), 4x daily for 3 days. Splenocytes were  
20           analyzed for CD11b expression by FACS (C), assessed for their osteoclastogenic potential by TRAP assay and CFU-M forming potential by colony assay (D). The data are presented as the mean  $\pm$  SEM of 4 replicate samples (\*p<0.005) from a representative mouse. Similar data were obtained from 2 additional mice.

          25. Figure 18 shows that Enbrel® (etanercept) reduces bone marrow edema in PsA. Enhanced bone marrow edema (EBME) was determined by Gd-enhanced fat suppressed MRI in 13 of 20 patients, scored by 2 independent radiologists on a 0-9 point scale. EMBE was assessed at screening (Baseline) and again following 6 months of Enbrel® etanercept therapy. The means are illustrated by the X. Improvement was significant (p $\leq$ 0.002). The change in EBME correlated with the decrease in OCP;  
30           R<sup>2</sup>=0.26.

          26. Figure 19 shows EBME as determined by Gd-enhanced fat suppressed MRI in 13 of 20 patients, scored by 2 independent radiologists on a 0-9 point scale. EMBE

was assessed at screening and again following 6 months of entanercept therapy. The means are illustrated by the X. Improvement was significant ( $p \leq 0.002$ ). The change in EBME correlated with the decrease in OCP;  $R^2 = .26$ .

27. Figure 20 shows that polyethylene wear correlates with peri-acetabular osteolysis. The osteolytic volume (in  $\text{cm}^3$ ) is plotted as a function of the volume of polyethylene wear (in  $\text{mm}^3$ ) for 20 patients with peri-acetabular osteolysis of an uncemented cup (C). Of note are 3 patients that are resistant to osteolysis (box).

28. Figure 21 shows a SLE-TNF transcriptome. (A) Hierarchical clustering of gene expression data by blood leukocytes of 9 healthy children, 30 with SLE, and 12 with juvenile chronic arthritis including 3 systemic arthritis. The SLE patients have been ranked according to their SLEDAI at time of blood draw. Each row represents a separate gene and each column a separate patient. 374 transcript sequences have been selected which were differentially expressed in SLE by comparison to healthy patients. The normalized expression index for each transcript sequence (rows) in each sample (columns) is indicated by a color code. Gray, light gray, and dark gray squares indicate that expression of the gene is greater than, equal to or less than the mean level of expression across 9 healthy controls. The scale extends from fluorescence ratios of 0.25 to 4.0. (B) Active SLE patients leukocytes display 36 IFN-up-regulated and 13 down-regulated transcript sequences. The same genes are altered in healthy PBMCs cultured in vitro with IFN. Median expression and the number of patients who display more than twofold increase in gene expression. \*\* Significant after Bonferroni correction, \* significant after Benjamini and Hochberg correction. (C) Extinction of IFN signature after steroid infusion. Analysis of PBMCs from 3 patients (#30, 25, and 5, see online Table S1) before and after (1–4 d) treatment with high dose intravenous GC (1g/day for 3 d). All patients show down-regulation of IFN-regulated genes. P values on the right indicate significance of the gene expression level before and after GC (paired  $t$  test).

29. Figure 22 shows systemic TNF $\alpha$  increases CD11b $^+$  osteoclast progenitors in spleen and blood. (A) Splenocytes and PBMC from five hTNF-Tg mice and their wild-type littermate controls were cultured for 5 days in 10ng/ml M-CSF and 100ng/ml RANKL, then fixed and stained for TRAP. The number of TRAP $^+$  multinucleated cells per well was determined and the means  $\pm$  SEM are presented ( $*p < 0.005$ ). (B) To phenotype these OCP FACS analysis was performed with a variety of surface markers.

The most reliable proved to be CD11b as all of the OCP are in the CD11b<sup>hi</sup> population, as determined by cell sorting followed by TRAP osteoclastogenesis assay, and TNF-Tg splenocytes have a significant increase in this population.

30. Figure 23 shows that TNF $\alpha$  dysregulation leads to an increase in CD11b<sup>+</sup> osteoclast progenitors that is reversible with anti-TNF therapy. (A-C) hTNF-Tg mice  
 5 were treated with 10 mg/kg of Enbrel® (etanercept) or placebo i.p. twice a week for 2-week. Afterwards, splenocytes were isolated from these mice, together with untreated WT controls, and analyzed for (A) CD11b expression by FACS, (B) osteoclastogenic potential by TRAP assay, and (C) CFU- $\gamma$ M colony forming potential by methocellulose  
 10 plating. (A) Data from a representative mouse (n = 3). (B and C) The data are presented as the mean  $\pm$  SEM of 4 replicate samples (\*p<0.005 vs. WT, \*\*p<0.01 vs. PBS) from a representative mouse. Similar findings were also obtained from wild-type mice injected with 10ug of TNF for three days (D-F).

31. Figure 24 shows that TNF-Tg mice in a RANK null background have  
 15 osteopetrosis due to the absence of mature osteoclasts but still have an increase in OCP. Four-month-old TNF-Tg x RANK<sup>-/-</sup> mice were obtained by crossing TNF-Tg and RANK knockout mice. Splenocytes from these mice were stained for CD11b and analyzed by FACS. The data are presented as representative animals (n = 3 per group).

32. Figure 25 shows that a high dose RANK blockade effectively reduces  
 20 osteoclast numbers and prevents erosive arthritis in hTNF-Tg mice, but does not affect OCP frequency. 4-month-old hTNF-Tg mice with established arthritis were treated with 10 mg/kg of RANK:Fc or placebo i.p. daily for 2-week. After sacrifice the knee joints were prepared for histology, stained for TRAP activity and counter stained with hematoxylin/fast-green. Osteoclast numbers in the tibia (remodeling) and at the  
 25 pannus-bone junction (erosion) of the hTNF-Tg mice were quantified by histomorphometry (A). A similar analysis was performed with H&E and TRAP stained sections to evaluate bone erosion, which was done by dividing the eroded surface in mm by the total surface of the proximal tibia and distal femur (C). The spleens of these mice were also taken at the time of sacrifice and CD11b expression was determined by  
 30 FACS (B). The data are presented as a representative animal or the mean  $\pm$  SEM (\*p<0.01) of the group (n = 5).

33. Figure 26 shows TNF-induced OCP in PsA. PBMC from a healthy donor or PsA patient were culture with and without M-CSF and RANKL on glass slides or on cortical bone wafers. After the culture period the slides were stained for TRAP to visualize multinucleated osteoclasts, and the bone wafers were stained with toluidine blue to visualize resorption lacunae. (B-D) PBMC from PsA patients before and 12 weeks after etanercept therapy were assayed for OCP frequency via TRAP osteoclastogenesis assay (A) or CD11b expression by FACS (C & D).

34. Figure 27 shows that RANKL priming inhibits macrophage activation. (A) RAW cells were grown for 2 days in the indicated dose of RANKL and then stimulated with the indicated doses of IFN- $\gamma$  for 24hr. The culture supernatants were then analyzed for NO via Greiss reaction. (B) RAW cells were grown in media for 3 days (CTL), media for 2 days and stimulated with IFN- $\gamma$  for 24hr (IFN- $\gamma$ ), or RANKL for 2 days and stimulated with IFN- $\gamma$  for 24hr (RANKL+IFN- $\gamma$ ) and analyzed for CD11b (mean fluorescence intensity and RANK (% pos.) expression by FACS.

35. Figure 28 shows TNF-induced OCP in mice and man. Splenocytes from TNF-Tg and non-Tg littermate controls (WT) mice (left) and PBMC from healthy donors and PsA patients (right) were analyzed for CD11b expression by FACS.

36. Figure 29 shows a schematic model of mutually-exclusive osteoclast, macrophage and dendritic cell differentiation. The central hypothesis of this program is that a chronic-systemic innate immune response drive by the TNF/IFN axis dominantly regulates the erosive phenotype of inflammatory arthritis by altering myelopoiesis towards osteoclast and dendritic cell differentiation in a mutually exclusive fashion. This process starts following the release of an early Myeloid Precursor that has left the granulocyte lineage (Gr1-), and is released from the bone marrow into the blood. Following entry into the end-organ this cell will receive a signal to terminally differentiate into an osteoclast, activated macrophage, or a dendritic cell in a mutually exclusive fashion.

37. Figure 30 shows a SLE-TNF transcriptome. (A) Hierarchical clustering of gene expression data by blood leukocytes of 9 healthy children, 30 with SLE, and 12 with juvenile chronic arthritis including 3 systemic arthritis. The SLE patients have been ranked according to their SLEDAI at time of blood draw. Each row represents a separate gene and each column a separate patient. 374 transcript sequences have been

selected which were differentially expressed in SLE by comparison to healthy patients. The normalized expression index for each transcript sequence (rows) in each sample (columns) is indicated by a color code. Red, yellow, and blue squares indicate that expression of the gene is greater than, equal to or less than the mean level of expression across 9 healthy controls. The scale extends from fluorescence ratios of 0.25 to 4.0. (B) Active SLE patients leukocytes display 36 IFN-up-regulated and 13 down-regulated transcript sequences. The same genes are altered in healthy PBMCs cultured in vitro with IFN. Median expression and the number of patients who display more than twofold increase in gene expression. \*\* Significant after Bonferroni correction, \* significant after Benjamini and Hochberg correction. (C) Extinction of IFN signature after steroid infusion. Analysis of PBMCs from 3 patients (#30, 25, and 5, see online Table S1) before and after (1–4 d) treatment with high dose intravenous GC (1g/day for 3 d). All patients show down-regulation of IFN-regulated genes. P values on the right indicate significance of the gene expression level before and after GC (paired *t* test).

#### IV. DETAILED DESCRIPTION

38. Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods or specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

##### A. Definitions

39. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

40. Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value.

Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both

in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that when a value is disclosed that “less than or equal to” the value, “greater than or equal to the value” and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value “10” is disclosed the “less than or equal to 10” as well as “greater than or equal to 10” is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point “10” and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15.

41. In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

42. “Optional” or “optionally” means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

43. Primers are a subset of probes which are capable of supporting some type of enzymatic manipulation and which can hybridize with a target nucleic acid such that the enzymatic manipulation can occur. A primer can be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art which do not interfere with the enzymatic manipulation.

44. Probes are molecules capable of interacting with a target nucleic acid, typically in a sequence specific manner, for example through hybridization. The hybridization of nucleic acids is well understood in the art and discussed herein. Typically a probe can be made from any combination of nucleotides or nucleotide derivatives or analogs available in the art.

45. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this

pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

46. Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular inflammatory joint disease is disclosed and discussed and a number of modifications that can be made to a number of molecules including the inflammatory joint disease are discussed, specifically contemplated is each and every combination and permutation of inflammatory joint disease and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

#### **B. Compositions and Methods**

47. Inflammatory arthritis is a prominent clinical manifestation in diverse autoimmune disorders including rheumatoid arthritis (RA), psoriatic arthritis (PsA), systemic lupus erythematosus (SLE), Sjogren's Syndrome and polymyositis. Most of these patients develop joint deformities on physical examination but typically only RA and PsA patients manifest bone erosions on imaging studies.



48. Chronic inflammatory bone diseases, such as rheumatoid arthritis (RA), are accompanied by bone loss around affected joints due to increased osteoclastic resorption. This process is mediated largely by increased local production of pro-inflammatory cytokines (Teitelbaum SL. *Science* 2000; 289(5484):1504-1508; Goldring SR and Gravallesse EM. *Arthritis Res* 2000; 2(1):33-7). These cytokines can act directly on cells in the osteoclast lineage or indirectly by affecting the production of the essential osteoclast differentiation factor, receptor activator of NF $\kappa$ B ligand (RANKL), and/or its soluble decoy receptor, osteoprotegerin (OPG), by osteoblast/stromal cells (Hofbauer LC, et al. *J Bone Miner Res* 2000; 15(1):2-12). TNF $\alpha$  is a major mediator of inflammation, whose importance in the pathogenesis of various forms of bone loss is supported by several lines of experimental and clinical evidence (Feldmann M, et al. *Cell* 1996; 85(3):307-10). However, TNF $\alpha$  is not essential for osteoclastogenesis (Douni E, et al. *J. Inflam.* 1996; 47:27-38), erosive arthritis (Campbell IK, et al. *J Clin Invest* 2001; 107(12):1519-27), or osteolysis (Childs LM, et al. *J. Bon. Min. Res.* 2001; 16:338-347), as these can occur in the absence of TNF $\alpha$ . The critical question of how TNF $\alpha$  increases osteoclastogenesis *in vivo* remains to be answered.

49. Disclosed herein, Psoriatic arthritis (PsA) is an inflammatory joint disease characterized by extensive bone resorption. Also disclosed herein, blood samples from PsA patients, particularly those with bone erosions on plain radiographs, exhibit a marked increase in osteoclast precursors (OCP) compared to healthy controls. Additionally disclosed, PsA PBMC readily formed osteoclasts *in vitro* without exogenous RANKL or M-CSF, and both osteoprotegerin (OPG) and anti-tumor necrosis factor (TNF) antibodies inhibited osteoclast formation. Additionally, cultured PsA PBMC spontaneously secreted higher levels of TNF- $\alpha$  than healthy controls. *In vivo*, OCP frequency declined substantially in PsA patients following treatment with anti-TNF agents. Immunohistochemical analysis of subchondral bone and synovium revealed RANK positive perivascular mononuclear cells and osteoclasts in PsA specimens. RANKL expression was dramatically upregulated in the synovial lining layer while OPG immunostaining was restricted to the endothelium. These results indicate a model for understanding the pathogenesis of aggressive bone erosions in PsA. OCP arise from TNF- $\alpha$  activated peripheral blood mononuclear cells that migrate

to the inflamed synovium and subchondral bone where they are exposed to unopposed RANKL and TNF- $\alpha$ . This leads to osteoclastogenesis at the erosion front and in subchondral bone resulting in a bi-directional assault on psoriatic bone.

50. In general, the efficacy of anti-TNF biological therapies (Enbrel®  
5 (etanercept), infliximab and adalimumab) in rheumatoid and psoriatic arthritis, psoriasis and ankylosing spondylitis is assessed three months after beginning treatment. Response is traditionally defined in terms of clinical improvement (decrease in the number of tender and swollen joints, decline in the Psoriasis Assessment Severity Index and improved patient and global assessment) and a decline in the level of the  
10 erythrocyte sedimentation rate and/or the C-reactive protein level. Due to the high cost of anti-TNF therapies, patients who fail to reach the endpoints outlined above are frequently deemed non-responders and treatment is terminated.

51. Based on data from the clinical trial and experience in the clinic, it was found that these endpoints are inadequate, since a significant number of “non-  
15 responders” ultimately achieve these endpoints following longer courses of therapy. Thus, the biomarker used herein, the frequency of circulating osteoclast precursors (OCP), can be an invaluable tool for rapidly screening potential responders from non-responders. An extremely high correlation in psoriatic arthritis patients between improved clinical response, lessening bone marrow edema on MRI (a known predictor  
20 of subsequent bone damage) and the decline in OCP frequency after treatment with Enbrel® etanercept has been observed. Hence, OCP frequency can be a useful biomarker for predicting which patients with Crohn’s disease, psoriasis, rheumatoid arthritis and ankylosing spondylitis will respond to anti-TNF therapy.

52. Disclosed herein are methods and compositions for diagnosing, monitoring,  
25 and treating inflammatory joint diseases, as well as compositions for the same. Many inflammatory joint diseases are discussed herein including arthritis, as discussed herein, including rheumatoid arthritis (RA) and psoriatic arthritis (PsA), and systemic lupus erythematosus (SLE), Sjogren’s Syndrome, and polymyositis as discussed herein. In certain embodiments the disclosed methods revolve around osteoclast precursor cells  
30 (OCPs) and markers related to these cells because the presence of the cells, as discussed herein, is associated with the presence and severity of inflammatory joint diseases. Furthermore, in certain embodiments the methods revolve around peripheral blood

mononuclear cells (PBMCs) and assaying samples of PBMCs for the presence of OCPs.

As discussed herein there are many markers for OCPs and PBMCs which can be used in the methods, as well as many different diseases correlated with the presence of OCPs. Furthermore, any method for monitoring the disclosed cells can be used including those specifically disclosed herein. Additionally, there are many different signaling molecules, as disclosed herein, related to the activation, presence, and persistence of inflammatory joint diseases. For example, TNF, such as TNF- $\alpha$ , and its cognate receptors, such as TNF-R1 and TNF-R2, RANKL, and its cognate receptor RANK, as well as OPG and IFN and their cognate receptors can be involved in the disclosed methods and diseases. It is understood that the disclosed methods can incorporate the characteristics of these and other molecules. For example, molecules that bind to these molecules and inhibit their function can be used in the disclosed methods. Furthermore, it is understood that the discussion of arthritis, osteoclasts and osteoclast precursors, as well as other cells, the role of certain cells in diseases of joint inflammation, such as PSA and RA, as well as the disclosed molecules and various cellular and animal, such as mouse, models of the diseases discussed herein, as well as general characteristics, such as sequence similarities nucleotides and proteins and peptides and expression systems for the same, and antibodies and pharmaceutical compositions and delivery techniques, as well as any other concepts discussed herein, can be incorporated in the disclosed methods and compositions.

### 1. Arthritis

53. Inflammatory arthritis is one of the most common clinical features in autoimmune disorders manifesting as pain, stiffness and inflammation. Psoriatic Arthritis is characterized by chronic, autoimmune inflammatory arthritis, and can be associated with psoriasis. Often PsA patients are seronegative for rheumatoid factor. Clinical Features of PsA include progressive and destructive, joints are less painful and tender, joints are less swollen and erosions and fusion can occur early and rapidly. There are cytokines in PsA synovium. For example, IL-2, IFN- $\gamma$ , and IL-10 present at levels significantly higher than in RA (Ritchlin,C., et al. 1998. *J Rheumatol* 25:1544-1552), and IL-1 $\beta$  and TNF are also present at high levels (Ritchlin,C., et al. 1998. *J Rheumatol* 25:1544-1552; Danning,C.L., et al. 2000. *Arthritis Rheum.* 43:1244-1256).

54. PsA can be distinguished from RA on the basis of unique clinical features, the absence of rheumatoid factor and characteristic radiographic findings( Winchester,R. 1993. Psoriatic Arthritis. In *Dermatology in General Medicine*. T.B.Fitzpatrick, et al., editors. 515-527). Patients frequently develop focal

5 inflammation at multiple sites involving skin, joints and tendon insertion sites or entheses (Helliwell,P., et al. 1991. *Brit J of Rheuma* 30:339-345 ). Radiologic evidence of joint damage (erosions and joint space narrowing) is observed in almost all patients with rheumatoid arthritis (RA), followed for more than five years ( Kaarela, K., et al. 1993. *J Rheumatol* 20:1670; Kaarela, K., and S. Sarna. 1993. *Clin Exp Rheumatol*

10 11:643). Joint damage is also very common in psoriatic arthritis (PsA), an inflammatory joint disease that occurs in 10-15% of psoriasis patients ( Mease, P. J. 2002. *Ann Rheum Dis* 61:298). Gladman and colleagues noted that two-thirds of PsA patients had bone erosions on initial presentation to a rheumatologist ( Gladman, D. D., et al. 1995. *J Rheumatol* 22:675). Radiographs often show extensive bone loss manifesting as

15 eccentric erosions, frank tuft resorption and pencil-in cup deformities and acrololysis (Gladman, D.D. 1998. *Psoriatic arthritis. Rheumatic Diseases Clinics of North America* 24:829-844; Resnick,D. and Niwayama,G. 1981. Psoriatic Arthritis. In *Diagnosis of Bone and Joint Disorders*. D.Resnick and Niwayama,G., editors. 1103; Resnick, D., and G. Niwayama. 1989. In Resnick (Ed) *Bone and Joint Imaging*

20 Philadelphia: WB Saunders:320.). Histopathologically, many PsA patients have aggressive synovitis with marked synovial hyperplasia, extensive vascular proliferation with a tortuous morphology and pannus tissue penetrating deep into cartilage and bone ( Fearon, U., K. et al. 2003. *J Rheumatol* 30:260). In PsA, periarticular bone mineralization is maintained and there is often concomitant new bone formation in the

25 form of periostitis and frank ankylosis, findings not seen in RA ( Resnick,D. and Niwayama,G. 1977. *American J of Roentgenology*. 129:275-278; Bywaters,E.G. and Dixon,A.S. 1965. *Annals of the Rheum.Dis.* 24:313-331). The presence of marked bone resorption coupled with adjacent new bone formation (often in the same digit) suggests a disordered pattern of bone remodeling in the psoriatic joint. In addition, osteoclasts

30 are prominently situated at the bone-pannus junction and in bone marrow-derived cutting cones traversing the bone matrix, which is consistent with the bi-directional

attack originally described by Wooley in severely affected RA joints ( Bromley, M., et al. 1985. *Ann Rheum Dis* 44:676).

55. Despite the presence of joint deformities on physical examination, not all forms of inflammatory arthritis degrade cartilage and resorb bone. For example, SLE is  
 5 a systemic autoimmune disorder with diverse clinical manifestations, but musculoskeletal symptoms are usually the chief complaint with 50% of patients reporting articular pain on presentation ( Kaposi, M. 1972. *Arch Dermat u Syph* 4:36; Stevens, M. 1983. In: *Shur PH, ed New York: Grune & Stratton:63*). Lupus patients often develop rheumatoid-like deformities (ulnar deviation, tendonopathies and  
 10 subluxation) but only 4-6% of patients display erosive changes on plain radiographs ( Esdaile, J. M., et al. 1981. *Ann Rheum Dis* 40:124; Grigor, R., et al. 1978. *Ann Rheum Dis* 37:121). Histopathologic analysis of SLE synovium shows mild to moderate synovial hyperplasia, microvascular changes and perivascular inflammation with mononuclear cells ( Goldenberg, D. L., and A. S. Cohen. 1978. *Medicine (Baltimore)*  
 15 57:239; Labowitz, R., and H. R. Schumacher, Jr. 1971. *Ann Intern Med* 74:911; Natour, J., et al. 1991. *Clin Exp Rheumatol* 9:221). Notably, aggressive synovial tissue invading cartilage or bone is not described in these studies. Indeed, Bywaters noted the similarity of the SLE joint pattern to that reported by Jaccoud in rheumatic fever. In Jaccoud's arthropathy, joint deformities are manually reducible and radiographs do not  
 20 depict bone or joint damage. In one large study of 939 SLE patients, Jaccoud's arthropathy was identified in 43% of the patients and was associated with a benign prognosis ( Molina, J. F., et al. 1995. *J Rheumatol* 22:347). Hand radiographs from a patient with SLE and PsA show that both patients demonstrated deformities of digits on physical exam. However, only the PsA patient displayed marked erosive changes in the  
 25 proximal and distal joints in both hands. Radiographs from the SLE patient showed no bone erosions or joint space narrowing.

56. Periprosthetic osteolysis as it relates to aseptic loosening of total hip replacements also involves inflammatory erosions at the joint space (Schwarz, E. M., et al. 2000. *Arthritis Res.* 2:165). Towards a solution to this problem, a volumetric CT  
 30 method to quantify osteolysis in patients with aseptic loosening was developed, and used it to publish the first study demonstrating the direct relationship between polyethylene wear and osteolysis ( Looney, R. J., et al. 2002. *Arthritis Res* 4:59). This

study demonstrated that some people are resistant to wear debris-induced osteolysis (Figure 20). Subsequently, a wear analysis was performed on an SLE patient who underwent a polyethylene liner replacement surgery due to excessive wear, as evidenced by the migration of the femoral component. Remarkably, while this patient had a wear rate that was well above average (885 mm<sup>3</sup> of polyethylene wear), there was no evidence of any periprosthetic bone loss. Considering the lack of surgical and material variables between this patient and a previous cohort, an innate resistance to osteolysis must be considered to best explain this observation.

## 2. Osteoclasts and osteoclast precursors (OCPs)

57. Osteoclasts, the principal cells responsible for bone resorption (Teitelbaum, S.L. 2000. *Science* 289:1504-1508), are multinucleated cells derived from mononuclear cell precursors of the monocyte/macrophage lineage (Massey, H.M. and Flanagan, A.M. 1999. *British Journal of Haematology* 106:167-170). Cell culture techniques (Suda T, et al. *J Bone Miner Res* 1997; 12(6):869-79) and transgenic and knockout mice (Karsenty G. *Genes Dev* 1999; 13(23):3037-51) have advanced the understanding of osteoclastogenesis and established that macrophage-colony stimulating factor (M-CSF) and RANKL are required for osteoclastogenesis (Lacey DL, et al. *Cell* 1998; 93(2):165-76; Yasuda H, et al. *Proc Natl Acad Sci U S A* 1998; 95(7):3597-602; Yoshida H, et al. *Nature* 1990; 345(6274):442-4; Kodama H, et al. *J Exp Med* 1991; 173(1):269-72; Kong YY, et al. *Nature* 1999; 397(6717):315-23; Dougall WC, et al. *Genes Dev* 1999; 13(18):2412-24; Dougall WC, et al. *Genes Dev* 1999; 13(18):2412-24). Osteoclastogenesis is also dependent on intracellular signaling molecules, including the adapter protein TRAF6 (Lomaga MA, et al. *Genes Dev* 1999; 13(8):1015-24; Kobayashi N, et al. *Embo J* 2001; 20(6):1271-1280), the transcription factors AP-1 (Wang ZQ, et al. *Nature* 1992; 360(6406):741-5; Johnson RS, et al. *Cell* 1992; 71(4):577-86) and NF- $\kappa$ B (Franzoso G, et al. *Genes Dev* 1997; 11(24):3482-96; Iotsova V, et al. *Nature Medicine* 1997; 3(11):1285-9), which are involved in mediating the M-CSF and RANKL signals (Anderson DM, et al. *Nature* 1997; 390(6656):175-9; Wong BR, et al. *J Biol Chem* 1997; 272(40):25190-4; Hsu H, et al. *Proc Natl Acad Sci U S A* 1999; 96(7):3540-5).

58. It has been proposed from experimental models that pathological resorption is at least, in part due to an increase in the number of these precursors (Li, P., et al.

2002. *Journal of Bone & Mineral Research* 17:s130). Indeed, elevated numbers of circulating osteoclast precursors (OCP) have been identified in the peripheral blood of patients with aggressive multiple myeloma and the bone marrow of patients with Paget's disease.( Gregoret,M.G., et al. 1995. *Leukemia* 9:1392-1397; Demulder,A., et  
 5 al. 1993. *Endocrinology* 133:1978-1982). Thus, investigation of the factors that promote osteoclast development can lead to control of events responsible for pathologic bone loss in PsA.

59. Homeostatic differentiation of osteoclasts or osteoclastogenesis is a contact dependent process directed by osteoblasts and stromal cells in the bone  
 10 microenvironment ( Lam,J., et al. 2000. *J of Clin. Inv.* 106:1481-1488; Suda,T., et al. 1999. *End Rev* 20:345-357). The osteoblasts/stromal cells release 2 different signals that are necessary and sufficient for differentiation of OCP into osteoclasts. The first, macrophage-colony stimulating factor (M-CSF) binds the receptor *c-fms* and the second, receptor activator of NF $\kappa$ B ligand (RANKL), binds to RANK on the surface of  
 15 OCP (Lacey,D.L., et al. 1998. *Cell* 93:165-176). Since permissive quantities of M-CSF are constitutively expressed in the bone microenvironment, it has been proposed that the relative expression of RANKL and its natural antagonist OPG ultimately control osteoclastogenesis (Teitelbaum,S.L. 2000. *Science* 289:1504-1508; Hofbauer,L.C. and Heufelder,A.E. 2001. *Arthritis Rheum.* 44:253-259; Nakagawa,N., et al. 1998. *Bioch &*  
 20 *Biophys Res Comm* 253:395-400; Simonet,W.S., et al. 1997. *Cell* 89:309-319). Furthermore, it has been demonstrated that miniscule quantities of RANKL are sufficient to synergize with TNF- $\alpha$  and potentiate osteoclastogenesis (Lam,J., et al. 2000. *J of Clin. Inv.* 106:1481-1488)

60. It has been found that TNF- $\alpha$  directly increases the number of OCP in mice  
 25 genetically modified to over-express TNF- $\alpha$  (hTNF-transgenic) and in normal mice injected with TNF- $\alpha$  (Li,P., et al. 2002. *Journal of Bone & Mineral Research* 17:s130). Additionally, treatment of hTNF mice with anti-TNF agents reduces the number of OCP to baseline. In humans, TNF- $\alpha$  levels are elevated in the psoriatic synovium and joint fluid (Ritchlin,C., et al. 1998. *J Rheum* 25:1544-1552; Partsch,G., et al. 1998.  
 30 *Ann.of Rheum.Dis.* 57:691-693; Danning,C.L., et al. 2000. *Arthritis Rheum.* 43:1244-1256). Disclosed herein osteoclast precursor frequency in PsA patients is increased, but it is not increased in healthy controls. The role of TNF- $\alpha$  and RANKL in promoting

osteoclast formation is also disclosed herein. Immunohistochemistry was performed on synovial tissues and bone obtained from patients with PsA and osteoarthritis (OA) to determine expression patterns of RANKL, RANK and OPG protein. The synovial expression of RANK, RANKL and OPG mRNA was also examined by RT-PCR.

5 Lastly, the ability of TNF- $\alpha$  to modulate OCP frequency in vivo was examined by determining the number of circulating OCP in PsA patients before and after anti-TNF therapy.

61. CD11b, CD14, CD51/61 and RANK are established markers of mononuclear OCP (Suda,T., et al. 1999. *End Rev* 20:345-357). It has been shown that  
 10 approximately 2% of PBMC can be stimulated to give rise to osteoclasts in vitro (Fujikawa,Y., et al. 1996. *Ann.of Rheum.Dis.* 55:816-822; Quinn,J.M., et al. 1998. *Endocrinology* 139:4424-4427). Interestingly, CD14<sup>+</sup> monocytes can also differentiate into dendritic cells and macrophages (Massey,H.M. and Flanagan,A.M. 1999. *British Journal of Haematology* 106:167-170), (Kotake,S., et al. 2001. *Arthritis Rheum.*  
 15 44:1003-1012). Presumably events in the bone marrow, circulation and possibly the synovium determine the fate of a particular monocyte. Indeed, following exposure to RANKL and M-CSF, a sub-population of monocytes rapidly loses the CD14 marker and acquires an osteoclast phenotype (Nicholson,G.C., et al. 2000. *Clin Science* 99:133-140) underscoring the critical importance of the RANK signaling pathway in  
 20 osteoclastogenesis.

### 3. PsA and osteoclasts

62. In psoriatic arthritis (PsA), bone erosions can be extensive, resulting in joint deformity and disability. These erosions differ markedly from the periarticular osteopenia and pericapsular bone loss commonly observed in rheumatoid joints, but  
 25 OCPs are increased in RA as well. (Martel,W., et al. 1965. *Radiology* 84:204). While these radiographic features suggest a different mechanism of bone loss in PsA, understanding the basis for this difference was impeded because the events that lead to psoriatic bone resorption have not been well defined. To elucidate this process, disclosed herein is how osteoclast precursors and the regulatory molecules RANK,  
 30 RANKL and OPG can orchestrate osteolysis in PsA.

63. The results disclosed herein demonstrate that osteoclasts are prominently situated at the bone-pannus junction and in cutting cones traversing the subchondral



bone in the psoriatic joint. In addition, osteoclast precursors are markedly increased in the circulation of PsA patients, most strikingly in those with bone erosions on plain radiographs. These cells express the surface markers CD11b, CD14, CD51/61 and RANK. A pivotal role for TNF- $\alpha$  in promoting OCP formation is supported by the observations that blocking TNF- $\alpha$  in vivo markedly suppressed the number of circulating OCP and that cultured PsA PBMC spontaneously release high quantities of biologically active TNF- $\alpha$ . Immunohistochemical studies delineated the presence of RANK positive cells in synovium and adjacent to blood vessels in subchondral bone. Furthermore, synovial lining cells stained strongly for RANKL while OPG expression was confined to the endothelium. These data indicate that OCP enter a synovial microenvironment characterized by a high ratio of RANKL to OPG expression, facilitating osteoclastogenesis and bone resorption.

64. Examination of bone marrow cultures from Paget's patients revealed an increase in the number of committed OCP compared to healthy controls (Demulder, A., et al. 1993. *Endocrinology* 133:1978-1982). Similarly, PBMC cultured from patients with multiple myeloma and bone lesions, but not those without bony involvement or healthy controls, gave rise to osteoclasts that resorbed bone in vitro when cultured in the presence of a murine stromal line (Gregoretti, M.G., et al. 1995. *Leukemia* 9:1392-1397). Faust et al. extended these observations by showing that osteoclasts can develop from unstimulated PBMC derived from healthy controls when grown at high density, however, the number of osteoclasts was not quantified and they demonstrated weak bone resorption properties (Faust, J., et al. 1999. *J Cell Biochem.* 72:67-80). In pilot studies, it was noted that numerous osteoclasts were present in unstimulated wells of PBMC cultured from PsA patients even when cultured at low density. Thus, the experimental protocol was modified, analyzing OCP frequency at low density in the absence of exogenous factors such as RANKL and M-CSF. Using this approach, osteoclasts were identified by positive TRAP staining and multinuclearity. These cells were shown to be functional by their ability to form pits on bone wafers. Compared to healthy controls, PsA patients had markedly more OCP and these cells resorbed significantly greater quantities of bone. The difference in resorption area between control and PsA patients was less than the difference in OCP frequency between the two groups. The additional finding that the increase in OCP frequency correlated with

clinical erosions indicates that the size of the precursor pool can be a dependent factor that contributes directly to bone resorption in PsA.

65. Studies in mice demonstrated that systemic TNF- $\alpha$  directly increases OCP frequency and that this elevation is reversible by anti-TNF therapy (Li,P., et al. 2002. *Journal of Bone & Mineral Research* 17:s130). Here it is shown that this is also true in PsA as increased OCP frequency declined significantly in 5 of 5 patients treated with anti-TNF therapy, which paralleled clinical improvement. Moreover, PsA PBMC spontaneously released high levels of TNF- $\alpha$  in vitro. TNF- $\alpha$  secreted by these cells promoted osteoclastogenesis that was blocked with anti-TNF- $\alpha$  antibodies. Blocking RANKL with OPG also substantially decreased the number of OCP that arose from PsA PBMC. Thus, the results imply that TNF- $\alpha$  triggers a systemic increase in the number of circulating OCP and this is consistent with it being a critical event in the modulation of psoriatic bone resorption. While these data strongly support the concept that TNF- $\alpha$  released by PsA PBMC could promote an increased OCP frequency, they do not establish these cells as the principal source of the TNF- $\alpha$ . Further studies designed to specifically address this question are required.

66. Osteoclasts have been detected in rheumatoid synovium (Gravallese,E.M., et al. 1998. *Am J Path* 152:943-951; Bromley,M. and Woolley,D.E. 1984. *Arthritis Rheum.* 27:968-975). Furthermore, while osteoclast numbers in PsA tissues were considerably greater than in OA samples, they were not significantly different from RA. First, circulating OCP can be higher in PsA than RA resulting in a more sustained assault on bone. Second, the ratio of RANKL to OPG can be significantly greater in patients with destructive PsA or, alternatively, levels of anti-osteoclastogenic factors such as; interferon- $\gamma$ , IL-12 or GM-CSF could be higher in the rheumatoid joint. Third, the striking increased vascularity and vessel tortuosity characteristic of PsA but not RA (Reece,R.J., et al. 1999. *Arthritis Rheum.* 42:1481-1484) can facilitate enhanced recruitment and entry of OCP into the joint. Finally, other pro-osteoclastogenic factors such as IL-1 can be present in greater quantities in PsA joints providing an additional osteoclast activation signal. In support of this latter mechanism is the observation that IL-1 was markedly elevated in psoriatic but not rheumatoid synovial explants obtained from patients with erosive joint disease (Ritchlin,C., et al. 1998. *J Rheum* 25:1544-1552).

67. Disclosed herein in pre-clinical studies utilizing the TNF-transgenic (TNF-Tg) mouse model of erosive arthritis, and clinical studies with PsA patients, a dominant role for monocytes effector cells in the perpetuation of these chronic autoimmune diseases was identified. Specifically, it was found that monocyte effector cells  
 5 activated by the receptor activator of NF $\kappa$ B ligand (RANKL)-RANK signaling pathway, differentiate into osteoclasts that mediate erosive arthritis in mice and humans. In contrast, mononuclear cells similarly isolated from SLE patients differentiate along a dendritic cell pathway. The mechanisms responsible for these divergent events arise in response to the dominance of tumor necrosis factor- $\alpha$  (TNF) in  
 10 PsA; and interferon- $\alpha$  (IFN) in SLE. Interestingly, there is a subset of patients that suffer from both inflammatory arthritis and SLE. While the vast majority of these patients do not have erosive disease, a minority develops erosions and joint space narrowing.

#### 4. The Role of Innate Immunity in Autoimmune Rheumatic 15 Diseases

68. Innate immune mechanisms play a central role in the establishment and maintenance of autoimmune diseases like inflammatory arthritis and SLE. Although the underling etiologic agents responsible for these diseases have yet to be defined, a general paradigm to explain how these conditions become chronic via an auto-  
 20 regulatory cytokine cascade has emerged. This model posits that effector cells are stimulated to produce cytokines involved in innate immune responses that systemically activate downstream target cells, which respond directly or indirectly by producing the same cytokines in a vicious cycle. Over the last decade, genetically manipulated animal models and both pre-clinical and clinical biological therapies have generated formal  
 25 proof of this model. What remains to be elucidated is how cytokines mediate organ specific pathology and if there is a fundamental relationship between the cytokine-induced genes in circulating cells and the phenotype of the end-organ pathology. These issues are addressed herein. Shown herein are two cytokines, tumor necrosis factor- $\alpha$  (TNF) and interferon- $\alpha$  (IFN), which are known to play a prominent role in  
 30 innate immunity and autoimmune inflammatory arthritis. The relationship between TNF and IFN in erosive arthritis towards the development of safer and more effective

therapeutic agents is disclosed herein as well as methods of monitoring treatment protocols.

### 5. TNF- $\alpha$

TNF- $\alpha$  is a pivotal cytokine in PsA. TNF $\alpha$  stimulates RANKL production  
 5 by stromal cells (Hofbauer LC, et al. Bone 1999; 25(3):255-9), T lymphocytes (Cenci S, et al. J Clin Invest 2000; 106(10):1229-37), B lymphocyte (Kanematsu M, et al. J Bone Miner Res 2000; 15(7):1321-9), and endothelial cell (Collin-Osdoby P, et al. J Biol Chem 2001; 276(23):20659-72), and M-CSF production by murine or human stromal cells (Srivastava S, et al. J Biol Chem 2001; 276(12):8836-40). In addition to this  
 10 indirect mechanism for osteoclastogenesis, TNF $\alpha$  also can stimulate osteoclastogenesis directly (Abu-Amer Y, et al. J Biol Chem 2000; 275(35):27307-10) and strongly synergizes with IL-1 and RANKL to promote osteoclast differentiation and activation (Kobayashi K, et al. J Exp Med 2000; 191(2):275-86; Azuma Y, et al. J Biol Chem 2000; 275(7):4858-64; Lam J, et al. J Clin Invest 2000; 106(12):1481-8). TNF $\alpha$  has  
 15 been isolated from psoriatic synovial fluid and psoriatic synovial explants release elevated levels of TNF- $\alpha$ , which were highest in patients with erosive arthritis (Ritchlin,C., et al. 1998. *J Rheum* 25:1544-1552; Partsch,G., et al. 1998. *Ann.of Rheum.Dis.* 57:691-693). Also, psoriatic synovial lining cells express TNF- $\alpha$  protein (Danning,C.L., et al. 2000. *Arthritis Rheum.* 43:1244-1256). Perhaps the most  
 20 convincing evidence stems from clinical trials showing that TNF alpha blockade dramatically ameliorates psoriatic joint pain and swelling, resulting in FDA approval of Enbrel® (etanercept) for PsA, the first drug to receive this indication ( Mease,P.J., et al. 2000. *Lancet* 356:385-390; Mease,P.J., et al. 1999. *Arthritis Rheum.* 42:377 (Abstr.)). Lastly, in a recent report, TNF inhibition improved clinical parameters of arthritis and  
 25 reversed abnormal MRI bone and soft tissue signals in spondyloarthropathy patients with active joint and enthesal inflammation (Marzo-Ortega,H., et al. 2001. *Arthritis Rheum.* 44:2112-2117).When looking at Tumor Necrosis Factor (TNF) alpha (TNF- $\alpha$ ) in Psoriatic Arthritis/Psoriasis, its levels are elevated in the synovium (Terajima. Arch Dermatol Res.1998;290:246-252. 2. Ettehad. Clin Exp Immunol. 1994;96:146-151.) and serum (Shiohara. J Am Acad Dermatol. 1992;27:568-574. ) in affected patients. The serum levels fluctuate with disease activity. TNF can also be found in active  
 30 plaques (Partsch. J Rheumatol. 1997;24:518-523 and Ritchlin. J Rheumatol.

1998;25:1544-1552.). There is also higher TNF bioactivity in involved vs uninvolved skin.

## 6. Monocyte differentiation pathways

71. Monocytes, pivotal effector cells of innate immunity, differentiate into dendritic cells (DC), macrophages or osteoclasts (OC) in response to environmental signals. Monocyte precursors stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) plus interleukin (IL)-4 yield dendritic cells, but form osteoclasts if exposed to receptor activator of NFκB ligand (RANKL) and monocyte colony stimulating factor (M-CSF). Treatment of monocytes with M-CSF alone produces tissue macrophages and IFN-γ or IL-6 treatment shifts monocyte precursor differentiation to macrophages rather than DC and OC (Delneste, Y., et al. 2003. *Blood*. 101:143). In vivo, these differentiation pathways can be altered by trauma, infection and inflammation. In trauma patients, differentiation is dramatically switched from DC to macrophages. Likewise, infection of human monocytes with live *Mycobacterium tuberculosis* interferes with differentiation to competent DC. DC yield was also diminished in pre-diabetic autoantibody-positive type 1 diabetes mellitus relatives compared to healthy controls following treatment with GM-CSF and IL-4 (Takahashi, K., et al. 1998. *J Immunol* 161:2629). IFN therapy is also known to bias monocyte differentiation in a manner that reduces osteoclast numbers in vivo (Thiele, J., et al. 1998. *Journal of Pathology*. 186:331). These data indicate that cytokines and possibly other signals released by cells and inflamed tissues can alter monocyte differentiation and effector cell function and have global effects on disease.

## 7. TNF in erosive arthritis

72. Experimental and clinical data demonstrating that TNF induces upregulation of other pro-inflammatory cytokines, adhesion molecules, matrix metalloproteinases, and oxygenases and potentiates osteoclastogenesis; places this cytokine at the apex of pro inflammatory cascade that leads to synovial inflammation and joint destruction (Feldmann, M., et al. 2003. *Blood* 101:143, Maini, R. N., et al. 1995. *Immunol Rev* 144:195). Of particular importance, however, there are two critical findings that for the material disclosed herein. First, TNF is necessary and sufficient to induce erosive arthritis in transgenic mice (TNF-Tg) (Keffer, J., et al. 1991. *Embo J* 10:4025), which is discussed in detail below. Second, multiple placebo controlled, double-blinded,

muticenter phase III clinical trials with three different FDA approved anti-TNF biologics: the soluble p75 TNF receptor-Fc fusion protein Enbrel® (etanercept) (Moreland, L. W., et al. 1997. *N Engl J Med* 337:141; Bathon, J. M., et al. 2000. *N Engl J Med* 343:1586), the chimeric anti-TNF monoclonal antibody infliximab (Lipsky, P. E., et al. 2000. *N Engl J Med* 343:1594, Braun, J., et al. 2002. *Lancet* 359:1187), and the humanized anti-TNF monoclonal antibody adalimumab (den Broeder, A., et al. 2002. *J Rheumatol* 29:2288, Weinblatt, M. E., et al. 2003. *Arthritis Rheum* 48:35), showed that ~70% of RA patients, who were refractory to standard DMARDs, improved physically, functionally and radiographically (Weinblatt, M. E., et al. 2003. *Arthritis Rheum* 48:35). Similarly, Enbrel® (etanercept) and infliximab have been remarkably effective in psoriatic arthritis. In phase II trials, both Enbrel® (etanercept) and infliximab treatment resulted in marked improvement in clinical outcome measures and Enbrel® (etanercept) inhibited joint destruction as early as six months after starting therapy (Mease, P. J. 2002. *Ann Rheum Dis* 61:298). Thus, studies in preclinical models and in patients with RA and PsA underscore the pivotal role of TNF- $\alpha$  in sustaining ongoing joint inflammation and destruction.

#### **8. The TNF $\alpha$ transgenic mouse (hTNF-Tg) model of chronic erosive arthritis**

73. Over the last two decades, several different animal models of arthritis have been developed, including adjuvant arthritis, streptococcal cell-wall arthritis, and antigen induced arthritis (Feldmann, M., et al. 2003. *Blood* 101:143). These models have provided valuable insights; however, each have limitations because the pathobiology of the arthritis differs from the synovitis and joint destruction observed in RA or PsA. More importantly, a major drawback of these models is that they represent acute synovitis facilitating evaluation of prophylactic but not long-term anti-inflammatory or immunosuppressive therapies. For these purposes, the hTNF $\alpha$ -transgenic mouse is an ideal model because the etiologic mechanism is evident and the chronic course of the synovitis and joint damage mirrors RA more closely. George Kollias' laboratory developed this animal model of arthritis by generating mice with a human TNF $\alpha$  transgene in which the AU rich 3' UT region (which shortens mRNA half life) was replaced by the stable  $\beta$  globin 3' UT, resulting in over-expression of TNF $\alpha$  (Keffer, J., et al. 1991. *Embo J* 10:4025). The transgene engenders multiple

lines of mice which develop polyarthritis. Anti-TNF $\alpha$  therapy alone completely prevents development of this disease. Macroscopic pathology includes swelling of the ankle joints at 6 to 8 weeks of age and impairment of movement. Beginning at 6 weeks of age and continuing throughout their life (>1year), hyperplasia of the synovial membrane, as well as cellular inflammatory infiltrates of the synovial space can be seen in nearly all joints examined. Pannus formation, articular cartilage destruction, and massive destruction of fibrous tissue are observed in the advanced stages of disease. These symptoms all closely mimic those of human disease. Interestingly, the inflammatory-erosive disease in hTNF-Tg mice is immune cell independent. These animals do not develop rheumatoid factor (Keffer, J., et al. 1991. *Embo J* 10:4025), and hTNF-Tg mice crossed with RAG-2 knockout mice (hTNF-Tg x RAG-/-), which have no T-cells or B-cells, suffer from arthritis that is almost indistinguishable from that of the parental strain ( Douni, E., K. et al. 1996. *J. Inflamm.* 47:27). Thus, consistent with the mission of this RFA, this model relies exclusively on innate immune mechanisms.

#### 9. RANK, RANKL, TNF- $\alpha$ TNF-R1, TNF-R2, and OPG

74. The discovery of RANK, RANKL and OPG as the final effector molecules ultimately regulating osteoclastogenesis and bone resorption has provided a fundamental insight into the mechanisms of osteolysis in metabolic bone diseases (Gori,F., et al. 2000. *Endocrinology* 141:4768-4776; Teitelbaum,S.L. 2000. *Science* 289:1504-1508; Gravallese,E.M. and Goldring,S.R. 2000. *Arthritis Res.* 2:33-37). Definitive proof in support of this paradigm has also been provided in animal models of inflammatory arthritis (Li,P., et al. 2002. *Journal of Bone & Mineral Research* 17:s130), (Fujikawa,Y., et al. 1996. *Ann.of Rheum.Dis.* 55:816-822; Quinn,J.M., et al. 1998. *Endocrinology* 139:4424-4427; Kotake,S., et al. 2001. *Arthritis Rheum.* 44:1003-1012). In RA, investigators found that RANKL mRNA is expressed by T lymphocytes and synoviocytes isolated from lining membranes Gravallese,E.M., et al. 2000. *Arthritis Rheum.* 43:250-258; Shigeyama,Y., et al. 2000. *Arthritis Rheum.* 43:2523-2530; Takayanagi,H., et al. 2000. *Nature* 408:600-605). It has also been demonstrated that fibroblast-like synoviocytes can induce osteoclastogenesis when co-cultured with PBMC (Takayanagi,H., et al. 1997. *Biochem. & Biophys. Res. Comm.* 240:279-286). In the immunohistochemistry experiments, it was found that PsA synovial lining cells stained intensely for RANKL, a finding not observed in OA synovial tissues. The

RANKL appeared to be relatively unopposed by OPG, since staining for this molecule was restricted to the endothelium. The likely targets for this synovial cell RANKL are the perivascular RANK positive mononuclear cells in the synovium and subchondral bone. The finding of RANK positive mononuclear cells in the synovium, confirmed by elevated RANK mRNA expression in at least some of the PsA patients, was in line with previous studies that detected TRAP positive cells in RA synovium and reported that osteoclasts can be generated from RA synovium and bone (Azuma, Y., et al. 2000. *J Bio Chem* 275:4858-4864; Gravallese, E.M., et al. 1998. *Am J Path* 152:943-951; Toritsuka, Y., et al. 1997. *J Rheumat* 24:1690-1696). A gradient of RANK staining was observed in mononuclear cells, increasing in intensity from the perivascular region in the subsynovium to the erosion front where synoviocytes and osteoclasts exhibited the strongest RANK expression. This gradient is directed by the elevated RANKL and TNF- $\alpha$  expressed by PsA synoviocytes. Ultimately, RANKL stimulation of these precursor cells could result in the genesis of RANK positive multinucleated osteoclasts that directly erode the bone matrix. Support for the critical role of RANKL is provided by the disclosed experiments indicating that OPG significantly blocked osteoclast formation in PsA PBMC.

**10. RANK, RANKL and OPG are the final effector molecules that control osteoclastogenesis and bone resorption.**

75. Osteoclasts are multinucleated cells formed by fusion of mononuclear progenitors derived from the monocyte/macrophage lineage ( Teitelbaum, S. L. 2000. Bone Resorption by Osteoclasts. *Science* 289:1504). Two distinct signals are necessary and sufficient for the differentiation of monocytes/macrophages into osteoclasts. The first is delivered by macrophage-colony stimulating factor (M-CSF), which signals through its receptor c-Fms. Receptor activator of nuclear factor kappa B ligand (RANKL) mediates the second through its receptor RANK. Cell culture conditions have been established in which M-CSF and RANKL are the only requirements for osteoclastogenesis ( Lacey, D. L., et al. 1998. *Cell* 93:165; Yasuda, H., et al. 1998. *Proc Natl Acad Sci U S A* 95:3597); and mice genetically deficient in M-CSF ( Yoshida, H., et al. 1990. *Nature* 345:442; Kodama, H., et al. 1991. *J Exp Med* 173:269) or RANKL ( Kong, Y. Y., et al. 1999. *Nature* 397:315; Dougall, W. C., et al. 1999. *Genes Dev* 13:2412) signaling are incapable of osteoclastogenesis and suffer from osteopetrosis.



Since M-CSF is constitutively expressed at permissive levels within the bone microenvironment it is believed that delivery of the RANK signal stimulates osteoclastogenesis *in vivo*. This event is negatively regulated by osteoprotegerin (OPG) (Simonet, W. S., et al. 1997. *Cell* 89:309), which is a natural decoy receptor that binds to RANKL blocking its interaction with RANK, and thereby inhibiting osteoclast development and activation (Teitelbaum, S. L. 2000. Bone Resorption by Osteoclasts. *Science* 289:1504). Disclosed herein, the ratio of RANKL to OPG is the major factor determining the extent of bone resorption in bone. In the revised paradigm of metabolic bone disease, RANKL and OPG are the final effector molecules that ultimately control osteoclastogenesis and bone resorption. Other factors modulate osteolysis (i.e. TNF) by altering the expression of RANKL and/or OPG ( Hofbauer, L. C., et al. 2000. *J Bone Miner Res* 15:2).

76. In RA, *RANKL* mRNA is up-regulated in T cells freshly isolated from the peripheral blood and synovium ( Shigeyama, Y., et al. 2000. *Arthritis Rheum* 43:2523; Goldring, S. R., and E. M. Gravallese. 2000. *Arthritis Res* 2:33). Additionally, synoviocytes isolated from rheumatoid synovium express *RANKL* mRNA and induce osteoclastogenesis when co-cultured with peripheral blood mononuclear cells (Gravallese, E. M., et al. 2000. *Arthritis Rheum* 43:250; Takayanagi, H., et al. 2000. *Arthritis Rheum* 43:259). Similarly, it was found that *RANKL*, is highly expressed in the synovial membrane adjacent to periarticular erosions and in stromal cells lining cutting cones in subchondral bone pointing to the pivotal importance of this molecule in the bi-directional attack on psoriatic bone. Although TNF is a potent stimulator of osteoclastogenesis (Abu-Amer, Y., et al. 2000. *J Biol Chem* 275:27307) and osteoclast activity (Bertolini, D. R., et al. 1986. *Nature* 319:516) it is not essential, since osteoclastogenesis ( Douni, E., K. et al. 1996. *J. Inflam.* 47:27), erosive arthritis (Campbell, I. K., et al. 2001. *J Clin Invest* 107:1519) and osteolysis (Childs, L. M., et al. 2001. *J. Bon. Min. Res.* 16:338), occur in its absence. Indeed, the specific role of TNF in osteoclastogenesis has been the subject of intense investigation. It has been shown that TNF is able to stimulate osteoclastogenesis indirectly through *RANKL* production by stromal cells (Hofbauer, L. C., et al. 1999. *Bone* 25:255), T cells (Cenci, S., et al. 2000. *J Clin Invest* 106:1229), B cells (Kanematsu, M., et al. 2000. *J Bone Miner Res* 15:1321) and endothelial cells (Collin-Osdoby, P., et al. 2001. *J Biol Chem*

276:20659). However, whether or not TNF $\alpha$  promotes osteoclast formation independently of RANK signaling is controversial. Two independent groups reported that TNF $\alpha$  promotes osteoclast formation *in vitro* under conditions of RANK blockade (Kobayashi, K., et al. 2000. *J Exp Med* 191:275; Azuma, Y., et al. 2000. *J Biol Chem* 275:4858) suggesting that TNF $\alpha$  can compensate for RANKL to induce osteoclastogenesis. This conclusion has been refuted by others who demonstrated that “permissive” levels of RANKL are required for TNF $\alpha$ -induced osteoclastogenesis (Lam, J., et al. 2000. *J Clin Invest* 106:1481). Furthermore, RANK independent osteoclastogenesis under physiological conditions *in vivo* has never been demonstrated. These latest observations are consistent with the murine studies of wear debris-induced osteolysis (Childs, L. M., et al. 2002. *J Bone Miner Res* 17:192), fracture healing, and erosive arthritis, where RANK blockade with 10mg/kg of RANK:Fc or genetic ablation of RANK completely eliminates all osteoclasts.

#### 11. Negative regulation of osteoclastogenesis and bone resorption by IFN.

77. Bone is an active tissue that is continually remodeled by osteoclasts and osteoblasts resulting in complete skeleton turnover every 10 years. Under normal homeostasis, this metabolic cycle is initiated by a decrease in blood calcium, which triggers parathyroid hormone (PTH) synthesis. PTH induces RANKL expression on stromal cells, which stimulates osteoclastic bone resorption and release of calcium. Once blood calcium levels normalize, PTH expression is extinguished and resorption is replaced by osteoblastic bone formation. Recently, Taniguchi and colleagues identified a novel autoregulatory loop. They found that the induction of type-I IFN (IFN- $\beta$ ) by RANKL delivers a critical signal to inhibit osteoclastogenesis (Takayanagi, H., et al. 2002. *Nature* 416:744). This was formally demonstrated with the IFN type-I receptor knockout mice (identical to the *Ifnr1*<sup>-/-</sup> mice disclosed herein) and IFN $\beta$ <sup>-/-</sup> mice, who demonstrate an increase in circulating OCP and enhanced osteoclastogenesis. These mice also have increased osteoclast numbers *in vivo* and develop severe osteopenia. They also demonstrated that direct administration of IFN $\beta$  together with LPS onto the calvaria of mice significantly inhibited bone loss, osteoclast formation and activation. The mechanism responsible for this autoregulatory loop was RANKL-induced cFos

expression in bone marrow monocytes, which leads to increased production of IFN $\beta$  that directly inhibits osteoclastogenesis in a paracrine fashion.

78. Type-II interferon (IFN $\gamma$ ) is also known to have potent anti-osteoclastic effects in vivo and in vitro. In similar studies, Takayanagi et al, utilized IFN $\gamma$  deficient mice to demonstrate that T-cell production of IFN $\gamma$  strongly suppresses osteoclastogenesis in bone marrow cultures by interfering with the RANKL-RANK signaling pathway. Furthermore, IFN $\gamma$  induced rapid degradation of the RANK adapter protein, TRAF6 (TNF receptor-associated factor 6) via ubiquitination, which resulted in strong inhibition of the RANKL-induced activation of the transcription factor NF $\kappa$ B and JNK. This inhibition of osteoclastogenesis was rescued by overexpressing TRAF6 in precursor cells, confirming that TRAF6 is the target critical for the IFN $\gamma$  action (Takayanagi, H., et al. 2000. *Nature* 408:600). These data were confirmed using a murine monocyte/macrophage cell line (RAW264.7) and primary murine splenocyte cultures (Huang, W., et al. 2003. *Arthritis Res Ther* 5:R49). However, in the models no evidence was found of IFN $\gamma$ -induced TRAF6 degradation or perturbations of NF $\kappa$ B, AP-1 or STAT signaling. Thus, it was concluded that these effects are more consistent with the cellular transformation that occurs during macrophage activation, which irreversibly differentiates monocytes out of the osteoclast lineage.

## 12. Interferon in SLE

79. Tissue damage resulting from antibody and complement-fixing immune complex deposition is one of the hallmarks of SLE. High titers of autoantibodies predominantly specific for DNA and nucleosomes, are among the immunologic features of SLE, leading to the hypothesis that inappropriate handling of dying (apoptotic) cells can represent a key pathogenic event (Mills, J. A. 1994. *N Engl J Med* 330:1871; Hahn, B. H. 1998. *N Engl J Med* 338:1359). Altered T-B cell interactions are thought to be a central mechanism leading to the loss of tolerance that characterizes SLE (Shlomchik, M. J., et al. 2001. *Nat Rev Immunol* 1:147), however, the recognition of DCs are key controllers of immunity (Steinman, R. M. 1991. *Annu Rev Immunol* 9:271) and tolerance (Steinman, R. M., et al. 2000. *J Exp Med* 191:411; Steinman, R. M., and M. C. Nussenzweig. 2002. *Proc Natl Acad Sci U S A* 99:351) led to the hypothesis that SLE can be driven by unabated DC activation. Over the last few years a new paradigm has emerged to explain the mechanism of unabated DC activation, which is primarily

regulated by systemic IFN $\gamma$  (IFN). This model is based on three fundamental observations: 1) CD14 $^{+}$  monocytes isolated from the blood of patients with systemic lupus erythematosus, but not those from healthy individuals, act as DCs. 2) These DCs appear to be activated by circulating IFN that is released by plasmacytoid DCs infiltrating lupus skin lesions. And most convincingly, 3) although only a fraction of patients with active SLE have detectable IFN levels in their serum, PBMC isolated from these patients express a unique IFN transcriptome, which reverts to a more normal pattern following effective steroid therapy (Baechler, E. C., et al. 2003. *Proc Natl Acad Sci U S A* 100:2610).

80. Figure 21 and 30, which are compilations of the microarray data disclosed herein (Baechler, E. C., et al. 2003. *Proc Natl Acad Sci U S A* 100:2610), demonstrate several important points. First, a single cytokine can exert systemic effects that dominantly alter monocyte activation. Second, the IFN-induced transcriptome in SLE PBMC is completely different from that observed in juvenile RA patients. This is consistent with the understanding that PsA patients with erosive disease can have a unique TNF-induced transcriptome in isolated PBMC that is consistent with differentiation towards the osteoclast rather than the dendritic cell lineage, as was observed in SLE.

### 13. Mouse models of SLE also demonstrate a systemic IFN disease

81. To understand the etiology of SLE and evaluate potential therapeutics, many labs have analyzed the hybrids of New Zealand black (NZB) and New Zealand white (NZW) mice. These mice develop severe immune complex-mediated glomerulonephritis associated with high serum levels of IgG antinuclear autoantibodies. As such, these mice are recognized as a model of human SLE. Based on the strong evidence indicating that the development of lupus in humans and in this murine model has a strong but complex genetic basis (Harley, J. B., et al. 1998. *Curr Opin Immunol* 10:690; Vyse, T. J., and B. L. Kotzin. 1998. *Annu Rev Immunol* 16:261; Theofilopoulos, A. N., and D. H. Kono. 1999. *Proc Assoc Am Physicians* 111:228; Wakeland, E. K., et al. 1999. *Curr Opin Immunol* 11:701), the identification of susceptibility genes has been a major focus of research. Specifically, much focus has been on the loci on distal chromosome 1, for which contributing genes from both the NZB and NZW have been localized (Vyse, T. J., and B. L. Kotzin. 1998. *Annu Rev*

*Immunol* 16:261; Wakeland, E. K., et al. 1999. *Curr Opin Immunol* 11:701; Morel, L., et al. 1994. *Immunity* 1:219; Kono, D. H., et al. 1994. *Proc Natl Acad Sci U S A* 91:10168; Drake, C. G., et al. 1995. *J Immunol* 154:2441; Rozzo, S. J., et al. 1996. *Proc Natl Acad Sci U S A* 93:15164; Rozzo, S. J., et al. 2000. *J Immunol* 164:5515;

5 Vyse, T. J., et al. 1997. *J Immunol* 158:5566; Vyse, T. J., et al. 1998. *J Immunol* 160:2757). In the laboratory, a NZB lupus-susceptibility locus on distal chromosome 1 (named *Nba2* for *New Zealand black autoimmunity 2*) has been apparent in multiple different backcrosses (Drake, C. G., et al. 1995. *J Immunol* 154:2441; Rozzo, S. J., et al. 1996. *Proc Natl Acad Sci U S A* 93:15164; Rozzo, S. J., et al. 2000. *J Immunol*

10 164:5515; Vyse, T. J., et al. 1997. *J Immunol* 158:5566; Vyse, T. J., et al. 1998. *J Immunol* 160:2757; Kotzin, B. L. 1996. *Cell* 85:303). In one cross, it was found that *Nba2*, in combination with the NZW MHC haplotype, accounted for >90% of the genetic contribution to IgG autoantibody production and disease (Rozzo, S. J., et al. 1996. *Proc Natl Acad Sci U S A* 93:15164). In different crosses, *Nba2* also showed

15 evidence for linkage as a quantitative trait locus with nearly all of the lupus autoantibodies studied, including IgG autoantibodies to chromatin, DNA, histones, and the retroviral envelope glycoprotein, gp70 (Vyse, T. J., et al. 1997. *J Immunol* 158:5566; Vyse, T. J., et al. 1998. *J Immunol* 160:2757). On the basis of these findings and enhancing effects on total IgG levels, it was understood that *Nba2* can act as an

20 immune response gene that influences antigen-driven B cell responses to self and possibly to exogenous antigens. More recently, mice congenic for the *Nba2* locus were generated and showed the contribution of this chromosomal region to autoantibody production and lupus nephritis (Rozzo, S. J., et al. 2001. *Immunity* 15:435). Expression profiling with oligonucleotide microarrays demonstrated differential expression of two

25 IFN-inducible genes, which both localized to the *Nba2* interval (Rozzo, S. J., et al. 2001. *Immunity* 15:435). The finding that these candidates are known IFN-inducible genes has led others to formally investigate the role of IFN- $\alpha$  in this model. Santiago-Raber et al examined the contribution of IFN by generating congenic NZB mice lacking the alpha-chain of IFN-alpha/betaR, the common receptor for the multiple IFN-

30 alpha/beta species. Compared with littermate controls, *Ifnar1*<sup>-/-</sup> x NZB mice had significantly reduced autoimmune symptoms, kidney disease, and mortality. These reductions were intermediate in the heterozygous-deleted mice. Most notably, the

disease-ameliorating effects were accompanied by reductions in dendritic cell maturation and T cell stimulatory activity, as disclosed herein. Based on these finding and the ability to use these mouse models in a pure C57B/6 background, TNF-Tg and Nba2 congenic mice can be used to test the action of *Nba2* as an immune response gene.

82. Collectively, the studies disclosed herein form a solid framework for understanding the cellular and molecular events responsible for the erosive phenotype in inflammatory arthritis. The observation that type I IFN inhibits osteoclastogenesis coupled with skewing of lupus monocyte differentiation down the dendritic cell pathway in SLE favors an IFN-based disease mechanism. In contrast, PsA is associated with a strong TNF signal that directs monocytes along an osteoclast pathway. Thus, the dominance of IFN in SLE patients provides a mechanism to explain the relative resistance of lupus patients to bone erosion.

#### 14. Methods of diagnosing

83. Disclosed are methods of diagnosing a subject with an inflammatory joint disease comprising measuring the number of osteoclast precursor cells (OCP) in the blood of the subject. Also disclosed are methods of determining the presence of an inflammatory joint disease in a subject comprising, obtaining a PBMC sample from the subject, and measuring the number of OCP in the PBMC of the subject, wherein a number of OCP in the PBMC of the subject greater than the number of OCP in a control subject indicates the presence of disease in the subject. Also disclosed are methods of diagnosing an inflammatory joint disease in a subject comprising, obtaining a sample from the subject and assaying the number of OCP in the sample, wherein an increase in OCP compared to a healthy control indicates an inflammatory joint disease in the subject.

84. An inflammatory joint disease refers to any condition in which cytokine release in an area of bone-bone or bone cartilage interaction results in the modification of healthy tissue, the development of synovitis i.e. transformation of the normal delicate synovial membrane into a hyperplastic inflamed tissue from its normal state. This modification can be destruction of healthy bone or cartilage tissue (e.g., bone resorption) or development. The result of this cytokine mediated modification is the inflammation of the joint which manifests as pain, swelling, itching, and heat. Examples

of inflammatory joint disease are psoriatic arthritis, rheumatoid arthritis, systemic lupus erythematosus (SLE), Sjogren's Syndrome and polymyositis. The disclosed methods and compositions could also be used for example to treat aseptic joint loosening of orthopedic implants, non-union of a fracture, spondyloarthropathies, psoriasis, and Crohns disease.

85. Typically osteoclast precursor cell refers to a circulating monocyte/macrophage lineage cell capable of osteoblast directed differentiation into osteoclasts. Two signals are necessary and sufficient for the differentiation process to occur 1) MCSF binds c-fms and 2) RANKL binds RANK on the osteoclast precursor cell surface.

86. A control subject refers to any individual that has not been diagnosed as having the disorder or condition being assayed. The terms "normal control" and healthy control" likewise mean a sample (e.g., cells, serum, tissue) taken from a source (e.g., subject, control subject, cell line) that does not have the condition or disorder being assayed and therefore may be used to determine the baseline for the condition or disorder being measured. Normal control and healthy control may also refer to samples taken from a source but not subject to a modification or stimulus that would result in the activation of differentiation of the sample. It is also understood that the control subject, normal control, and healthy control, include data obtained and used as a standard, i.e. it can be used over and over again for multiple different subjects. In other words, for example, when comparing a subject sample to a control sample, the data from the control sample could have been obtained in a different set of experiments, for example, it could be an average obtained from a number of healthy subjects and not actually obtained at the time the data for the subject was obtained.

87. Also disclosed are methods further comprising collecting the subject's PBMCs and methods wherein the step of measuring comprises counting the number of cells comprising at least one, two, three, four, five, six, seven, or eight markers selected from the group consisting of CD14, CD11a, CD11b, CD51/CD61, RANK, CCR1, CCR4, VCAM (CD106), VLA-4 (CD49d), and CD16, MHC Class II, B7.1, B7.2, CD40, c-fms. A marker has a well understood meaning in the art and can include for example, a molecule that is associated with a particular type of cells or set of types of cells, such as surface protein or expressed protein or lipid or carbohydrate. It is also

understood that markers can be assayed for their presence or absence, each of which can give information.

88. For example, disclosed are methods, wherein the step of measuring comprises counting the number of cells that are CD16- (negative).

5 89. Disclosed are methods, wherein the number of cells in the subject is compared to the number of cells obtained in a healthy control.

90. Disclosed are methods, wherein the amount of OCP is determined by staining the PBMC sample with fluorescently labeled antibodies for at least one marker selected from the group consisting of CD14, CD11b, CD51/CD61, RANK, CCR1,  
10 CCR4, VCAM (CD106), VLA-4 (CD49d), and CD16, CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms and visualizing the cells with labeled antibody bound to at least one of CD14+, CD11b+, CD51/CD61+, RANK+, CCR1+, CCR4+, VCAM+ (CD106), VLA-4+ (CD49d), CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms or CD16- using Fluorescence Activated Cell Sorting (FACS).

15 91. Herein, the terms “labeling” and “labeled” are used in reference to states of molecules that allow the molecules to be assayed. For example, molecules can be modified, labeled, such that the molecule can be visualized using detection methods well known in the art for radioactivity or fluorescence, for example. Examples of well known labels include but are not limited to fluorescent tags (e.g., Fluorescein  
20 isothiocyanate (FITC), Cascade Yellow, Cascade Blue®, Phycoerythrin (PE), PE-Texas Red®, Allophycocyanin (APC), Cy-5-PE, Cy-7-APC, and Peridinin Chlorophyll Protein (PerCP)), Biotin, Alkaline phosphatase, Horseradish peroxidase, and directly conjugated dyes.

92. Fluorescence activated cells sorting (FACS)” and “flow cytometry” are well  
25 understood methods in the art and include the ability to identify and mechanically sort cells based on whether the cell is labeled with a fluorescent label either directly or indirectly, i.e. a labeled molecule internalized by the cell or labeled molecule bound to the cell through binding to a marker on the cell.

93. Disclosed are methods wherein the number of OCP is determined by  
30 removing a tissue sample from the subject and visualizing the sample using immunohistochemistry for at least one marker selected from the group consisting of



CD14, CD11b, CD51/CD61, RANK, CCR1, CCR4, VCAM (CD106), VLA-4 (CD49d), CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms and CD16.

94. Tissue sample means any portion of an organism, organ, tissue, or cell that can be used for any diagnostic, screening, or testing purpose. Immunohistochemistry is well understood in the art and includes refers the use of labeled antibodies to directly visualize via microscopy (e.g., fluorescence microcopy, transmission electron microscopy, and light microscopy) the presence of features (ie., any cell structure, protein, glycoprotein, or marker to which an antibody may be directed) on or inside a given sample (e.g., tissue section or cells).

95. Disclosed are methods, wherein the amount of OCP is measured by removing a tissue sample from the subject and staining a tissue section with TRAP, counting the number of multinucleated cells, and comparing the number of multinucleated cells in the sample from the subject to the number of multinucleated cells in a sample from a healthy control, wherein more multinucleated cells in the sample from the subject than in the sample from the healthy control indicates an inflammatory joint disease in the subject.

96. Disclosed are methods, wherein the sample is a blood sample, wherein the sample is from the synovium of the subject, wherein the sample comprises bone marrow, and wherein the sample comprises perivascular mononuclear cells. Synovium is the delicate tissue that lines the joint space and is the primary site of inflammation in inflammatory arthritis and perivascular cells (cells that line the vasculature as it traverses in the synovium).

97. Disclosed are methods, wherein the amount of OCP in the subject's blood is measured using a colorimetric assay, and comparing the amount of OCP in the subject's blood to a standard curve. A standard curve has a well understood meaning and can refer to a set of samples used in an assay as a method of calibration among samples and separate assays. Also disclosed are methods, wherein the amount of OCP in the subject's blood is measured using a colorimetric assay, and comparing the amount of OCP in the subject's blood to the amount of OCP in a healthy control's blood. A colorimetric assay refers to any nonfluorescence assay where the results are quantified by measuring the amount of absorbance at a given wavelength of light. Such assays can include but are not limited to Enzyme-linked immunosorbance assays (ELISA).

98. Disclosed are methods, wherein the amount of OCP in the subject's blood is measured using FACS methods, Immunohistochemistry methods, Western methods, Southern methods, hybridization methods, RT-PCR methods, ELISA methods, ELISPOT methods, labeling methods, microarrays, bone wafer resorption, or  
5 Immunoprecipitation methods. These methods, as well as other detection methods, are well understood in the art. All of these methods, as well as others, are capable of, for example, identifying OCP cells, by for example, binding markers on OCP cells. For example, an Enzyme-linked immunospot (ELISpot) can refer to any assay in which the labeled antibodies are used to identify cells with a given characteristic, activity, or  
10 surface marker and the results are quantified by the visual enumeration of spots formed by the presence of antibody bound to a filter

99. Disclosed are methods, wherein the disease is psoriatic arthritis (PsA) or Rheumatoid arthritis (RA). Also disclosed methods designed to address, for example, aseptic joint loosening of orthopedic implants, non-union of a fracture,  
15 spondyloarthropathies, psoriasis and Crohns disease.

100. Disclosed are methods, wherein measuring the number of OCP comprises identifying RANK, CD11b and CD14 positive cells in the blood sample. There are numerous markers disclosed herein that are associated with OCP cells. For example, the presence of one or more CD14, CD11b, CD51/CD61, RANK, CCR1,  
20 CCR4, VCAM (CD106), VLA-4 (CD49d), CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms and the absence of CD16 are associated with OCPs. These markers can be identified using disclosed means, for example, through binding with labeled antibodies. Antibodies can be purchased, obtained, or made for these markers and others using standard means.

25 101. Disclosed are methods, wherein the subject shows bone erosion on a radiograph.

102. Also disclosed are methods of diagnosing an inflammatory joint disease comprising culturing peripheral blood mononuclear cells from a subject (PBMC) and assaying the number of osteoclasts formed.

30 103. Disclosed are methods, wherein assaying the number of osteoclasts formed comprises monitoring the amount of TRAP positive cells.

104. Disclosed are methods, wherein assaying the number of osteoclasts formed comprises monitoring the number of multinucleated cells, methods wherein an increased number of osteoclasts in the culture from the subject relative to the number of osteoclasts in a culture of PBMC from a control subject without joint inflammation  
5 disease indicates the subject has an inflammatory joint disease.

105. Disclosed are methods, wherein the culture has no exogenous RANKL or M-CSF added.

106. Disclosed are methods, wherein addition of RANKL or M-CSF increases the number of osteoclasts in the culture from the subject relative to the  
10 number of osteoclasts in a culture of PBMA from a control subject without an inflammatory joint disease, and wherein this increase indicates the subject has an inflammatory joint disease.

107. Disclosed are methods of diagnosing an inflammatory joint disease comprising culturing peripheral blood mononuclear cells (PBMC) from a subject and  
15 measuring the amount of TNF- $\alpha$  secreted.

108. Disclosed are methods of determining whether a subject has an inflammatory joint disease comprising, collecting PBMCs from the subject, allowing the PBMC to settle, fixing the cells, staining with anti-TRAP or TRAP activity, and examining the stained cells under a microscope, wherein the presence of TRAP  
20 indicates the subject has an inflammatory joint disease.

109. Also disclosed are methods of determining whether a subject has an inflammatory joint disease comprising isolating PBMC from the subject, and probing for a surface marker of mononuclear OCP. Probing is well understood in the art and can refer to using any method needed to identify the presence or absence of a marker.  
25 Disclosed are methods, wherein the surface marker comprises a marker selected from the group consisting of CD14, CD11b, CD51/CD61, RANK, CCR1, CCR4, VCAM (CD106), VLA-4 (CD49d), CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms and or CD16.

110. Disclosed are methods, wherein the surface markers are analyzed by  
30 FACS, methods wherein probing for a surface marker comprises assaying for at least one, two, three, four, five, six, seven, eight, or more surface markers.

111. Also disclosed are methods, wherein the subject is diagnosed with an inflammatory joint disease if the PBMC of the subject has more OCP than the PBMC of a control subject without an inflammatory joint disease.

112. Disclosed are methods of determining whether a subject has an inflammatory joint disease, comprising obtaining PBMC from the subject, culturing the PBMC on cortical bone wafers, and assaying the amount of eroded bone material in the cortical bone wafer. Cortical bone wafers can refer to thin slices of bovine bone that can be purchased commercially. Bone or other tissue is eroded when its density and composition is lessened.

113. Disclosed are methods, wherein the culturing occurs for any number of days including, for example, at least or greater than or equal to or less than or equal to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, or 50 days.

114. Disclosed are methods, wherein the subject is diagnosed with an inflammatory joint disease if the PBMC from the subject erodes more bone than the PBMC of a control subject.

115. Disclosed are methods of determining whether a subject has an inflammatory joint disease comprising assaying whether the osteoclasts of the subject express RANK.

#### **15. Method of following treatment claims**

116. Disclosed are methods of monitoring the treatment of subjects with an inflammatory joint disease using any of the disclosed methods herein. For example, disclosed are methods of monitoring the treatment for an inflammatory joint disease in a subject comprising, administering an anti-inflammatory disease agent to the subject, and measuring the number of osteoclast precursor cells (OCP) in the blood of the subject. Treatment refers to a method designed to reduce or alleviate the conditions associated with a disease or to diminish the disease or condition. It is specifically contemplated that treatment can include but is not limited to any change in the subject's disease state toward a non-diseased state. Treatment does not require the complete ablation of a disease or condition, but can include it. For example, a treatment can result in a 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, or 90

percent reduction in the amount of a symptom of a disease of inflammation of the joint in a subject.

117. An anti-inflammatory disease agent means any agent that results in the reduction of the symptoms or underlying disease that results in inflammation. For example, biologics, such as infliximab, Enbrel® (etanercept), adludimab, kinaret, 5 raptiva, osteoprotegerin (OPG), RANKFc, anti-RANKL, Bisphosphonates such as pamidronate, alendronate, actonel, zolendronate, clodronate and traditional DMARDS, such as methotrexate, azulfidine, hydroxychloroquine Corticosteroids-prednisone, methylprednisilone are anti-inflammatory disease agents, as well as salicylic acid, and 10 ibuprofen.

118. Disclosed are methods of monitoring the treatment for an inflammatory joint disease in a subject comprising, administering an anti-inflammatory disease agent to the subject, obtaining a PBMC sample from the subject, and measuring the number of OCP in the PBMC of the subject, wherein a decrease in the number of OCP in the 15 PBMC of the subject after treatment indicates the anti-inflammatory disease agent is having an effect on the disease.

#### **16. Method of treating claims**

119. Disclosed are methods of treating inflammatory disease, which involve assaying the subject to be treated using methods disclosed herein to diagnose the 20 presence of the inflammatory disease. For example, disclosed are methods of treating a subject with an inflammatory joint disease comprising measuring the number of OCP in the PMBC of the subject, and administering an anti-inflammatory disease agent if the number of OCP in the PMBC of the subject is greater than the number of OCP in the PBMC of a control subject.

120. Disclosed are methods, wherein the number of OCP are assayed a second time after the administration of the anti-inflammatory disease agent. 25

121. Also disclosed are methods, wherein the anti-inflammatory disease agent comprises OPG. The disclosed methods of treatment can use any any anti-inflammatory disease agent including Biologics such as infliximab, Enbrel® 30 (etanercept), adludimab, kinaret, raptiva, osteoprotegerin (OPG), RANKFc, anti-RANKL, Bisphosphonates, such as pamidronate, alendronate, actonel, zolendronate, clodronate and traditional DMARDS, such as methotrexate, azulfidine,

hydroxychloroquine Corticosteroids-prednisone, methylprednisilone. These therapies can be administered in any combination along with any other therapy, including for example physical therapy, or anti-inflammatory molecules such as acetiminophen or ibuprofren.

5           122. Disclosed are methods, where the anti-inflammatory disease agent comprises a composition that binds RANK, wherein the composition inhibits RANKL from binding RANK, wherein the anti-inflammatory disease agent comprises a composition that binds RANKL, wherein the composition inhibits RANK from binding RANKL, and wherein the composition is an antibody.

10           123. Also disclosed are methods wherein the anti-inflammatory disease agent comprises a composition that binds TNF-R1, wherein the antibody inhibits TNF- $\alpha$  from binding to TNF-R1, wherein the anti-inflammatory disease agent comprises a composition that binds TNF- $\alpha$ , wherein the antibody inhibits TNF-R1 from binding to TNF- $\alpha$ , and wherein the composition is an antibody. Also disclosed are methods  
15 wherein the anti-inflammatory disease agent comprises a composition that binds TNF-R2, wherein the antibody inhibits TNF- $\alpha$  from binding to TNF-R2, wherein the anti-inflammatory disease agent comprises a composition that binds TNF- $\alpha$ , wherein the antibody inhibits TNF-R2 from binding to TNF- $\alpha$ , and wherein the composition is an antibody.

20           124. Binds or binding refers to the interaction between two or more molecules beyond background or non-specific interaction. Inhibits or inhibiting refer to any reduction in a state, such as a disease, a binding interaction, or a condition. It is specifically contemplated that this action or result can include, but is not limited, to the complete ablation of the state.

25           125. Disclosed are methods, wherein the anti-inflammatory disease agent comprises an anti-TNF- $\alpha$  agent, and methods wherein the anti-TNF- $\alpha$  agent comprises, for example, Enbrel® (etanercept) or infliximab.

          126. Disclosed are methods of treating a subject with an inflammatory joint disease comprising administering an anti-inflammatory disease agent to the subject, and  
30 after administering the anti-inflammatory disease agent measuring the number of OCP in the PMBC of the subject, and adjusting the administration of the anti-inflammatory disease agent based on the number of OCP in the PBMC.

**17. Methods of selecting new anti-arthritic compounds**

127. As disclosed herein, the knowledge that the various molecules, such as RANK, RANKL, and TNF, and how they are related to the production of osteoclasts involved in inflammatory joint diseases, such as psoriatic arthritis, through the regulation of osteoclast precursors can be used to identify new therapeutics for the treatment of inflammatory joint diseases. For example, disclosed are methods of screening the efficacy of a pharmaceutical agent for the ability to treat an inflammatory joint disease comprising measuring the number of OCP in the PBMC of a subject, wherein the subject was administered the pharmaceutical agent, wherein a decrease in the number of OCP in the subject after treatment indicates efficacy of the pharmaceutical agent. Also disclosed are methods of identifying pharmaceutical agents having the ability to treat an inflammatory joint disease comprising measuring the number of OCP in a sample, assaying the number of OCP in sample from a non-treated control, and comparing the number of OCP in the subject and the non-treated control. Disclosed are methods, wherein the step of measuring comprises treating a PBMC sample with the agent, culturing the cells, and screening for cells comprising at least one marker selected from the group consisting of CD14, CD11b, CD51/CD61, RANK, CCR1, CCR4, VCAM (CD106), VLA-4 (CD49d), and CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms and CD16

**18. Kits for diagnosing joint disease**

128. 57. Disclosed are kits for diagnosing an inflammatory joint disease comprising any of the reagents discussed herein to perform, for example, the methods discussed herein. For example, disclosed are kits for diagnosing an inflammatory joint disease comprising reagents for identifying a marker selected from the group consisting of CD14, CD11b, CD51/CD61, RANK, CCR1, CCR4, VCAM (CD106), VLA-4 (CD49d), CD11a, MHC Class II, B7.1, B7.2, CD40, c-fms and and CD16, and a standard sample of a control subject without an inflammatory joint disease.

129. Disclosed are kits for diagnosing an inflammatory joint disease comprising reagents for identifying an OCP, and a standard sample of a control subject without an inflammatory joint disease. Also disclosed are for example, kits, wherein the reagent comprises a primer capable of hybridizing to the transcript of the marker,

kits wherein the reagent comprises a composition capable of binding to the marker, or wherein the composition comprises an antibody.

**C. Methods and reagents generally applicable to the disclosed methods and compositions**

5           130. There are a variety of methods and compositions disclosed herein, which might rely on, in a certain embodiment, an antibody, the hybridization of nucleic acids, a protein variant, a pharmaceutical composition, or methods of making recombinant cells, for example, to be screened for a therapeutic or produce a reagent. Disclosed is general and specific guidance for these and other methods, reagents, techniques, related  
10 to the disclosed compositions and methods.

**1. Sequence similarities**

131. It is understood that as discussed herein the use of the terms homology and identity mean the same thing as similarity. Thus, for example, if the use of the word homology is used between two non-natural sequences it is understood that this is  
15 not necessarily indicating an evolutionary relationship between these two sequences, but rather is looking at the similarity or relatedness between their nucleic acid sequences. Many of the methods for determining homology between two evolutionarily related molecules are routinely applied to any two or more nucleic acids or proteins for the purpose of measuring sequence similarity regardless of whether they are  
20 evolutionarily related or not.

132. In general, it is understood that one way to define any known variants and derivatives or those that might arise, of the disclosed genes and proteins herein, is through defining the variants and derivatives in terms of homology to specific known sequences. This identity of particular sequences disclosed herein is also discussed  
25 elsewhere herein. In general, variants of genes and proteins herein disclosed typically have at least, about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent homology to the stated sequence or the native sequence. Those of skill in the art readily understand how to determine the homology of two proteins or nucleic acids, such as genes. For example, the  
30 homology can be calculated after aligning the two sequences so that the homology is at its highest level.



133. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. MoL Biol.* 48: 443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

134. The same types of homology can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

135. For example, as used herein, a sequence recited as having a particular percent homology to another sequence refers to sequences that have the recited homology as calculated by any one or more of the calculation methods described above.

For example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using the Zuker calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by any of the other calculation methods. As another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using both the Zuker calculation method and the Pearson and Lipman calculation method even if the first sequence does not have 80 percent homology to the second sequence as calculated by the Smith and Waterman calculation method, the Needleman and Wunsch calculation method, the Jaeger calculation methods, or any of the other calculation methods. As yet another example, a first sequence has 80 percent homology, as defined herein, to a second sequence if the first sequence is calculated to have 80 percent homology to the second sequence using

each of calculation methods (although, in practice, the different calculation methods will often result in different calculated homology percentages).

## **2. Hybridization/selective hybridization**

136. The term hybridization typically means a sequence driven interaction  
5 between at least two nucleic acid molecules, such as a primer or a probe and a gene. Sequence driven interaction means an interaction that occurs between two nucleotides or nucleotide analogs or nucleotide derivatives in a nucleotide specific manner. For example, G interacting with C or A interacting with T are sequence driven interactions. Typically sequence driven interactions occur on the Watson-Crick face or Hoogsteen  
10 face of the nucleotide. The hybridization of two nucleic acids is affected by a number of conditions and parameters known to those of skill in the art. For example, the salt concentrations, pH, and temperature of the reaction all affect whether two nucleic acid molecules will hybridize.

137. Parameters for selective hybridization between two nucleic acid  
15 molecules are well known to those of skill in the art. For example, in some embodiments selective hybridization conditions can be defined as stringent hybridization conditions. For example, stringency of hybridization is controlled by both temperature and salt concentration of either or both of the hybridization and washing steps. For example, the conditions of hybridization to achieve selective  
20 hybridization may involve hybridization in high ionic strength solution (6X SSC or 6X SSPE) at a temperature that is about 12-25°C below the  $T_m$  (the melting temperature at which half of the molecules dissociate from their hybridization partners) followed by washing at a combination of temperature and salt concentration chosen so that the washing temperature is about 5°C to 20°C below the  $T_m$ . The temperature and salt  
25 conditions are readily determined empirically in preliminary experiments in which samples of reference DNA immobilized on filters are hybridized to a labeled nucleic acid of interest and then washed under conditions of different stringencies. Hybridization temperatures are typically higher for DNA-RNA and RNA-RNA hybridizations. The conditions can be used as described above to achieve stringency, or  
30 as is known in the art. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; Kunkel et al. *Methods Enzymol.* 1987:154:367, 1987 which is herein incorporated by

reference for material at least related to hybridization of nucleic acids). A preferable stringent hybridization condition for a DNA:DNA hybridization can be at about 68°C (in aqueous solution) in 6X SSC or 6X SSPE followed by washing at 68°C. Stringency of hybridization and washing, if desired, can be reduced accordingly as the degree of complementarity desired is decreased, and further, depending upon the G-C or A-T richness of any area wherein variability is searched for. Likewise, stringency of hybridization and washing, if desired, can be increased accordingly as homology desired is increased, and further, depending upon the G-C or A-T richness of any area wherein high homology is desired, all as known in the art.

138. Another way to define selective hybridization is by looking at the amount (percentage) of one of the nucleic acids bound to the other nucleic acid. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the limiting nucleic acid is bound to the non-limiting nucleic acid. Typically, the non-limiting primer is in for example, 10 or 100 or 1000 fold excess. This type of assay can be performed at under conditions where both the limiting and non-limiting primer are for example, 10 fold or 100 fold or 1000 fold below their  $k_d$ , or where only one of the nucleic acid molecules is 10 fold or 100 fold or 1000 fold or where one or both nucleic acid molecules are above their  $k_d$ .

139. Another way to define selective hybridization is by looking at the percentage of primer that gets enzymatically manipulated under conditions where hybridization is required to promote the desired enzymatic manipulation. For example, in some embodiments selective hybridization conditions would be when at least about, 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer is enzymatically manipulated under conditions which promote the enzymatic manipulation, for example if the enzymatic manipulation is DNA extension, then selective hybridization conditions would be when at least about 60, 65, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 percent of the primer molecules are extended. Preferred conditions also include those

suggested by the manufacturer or indicated in the art as being appropriate for the enzyme performing the manipulation.

140. Just as with homology, it is understood that there are a variety of methods herein disclosed for determining the level of hybridization between two nucleic acid molecules. It is understood that these methods and conditions may provide different percentages of hybridization between two nucleic acid molecules, but unless otherwise indicated meeting the parameters of any of the methods would be sufficient. For example if 80% hybridization was required and as long as hybridization occurs within the required parameters in any one of these methods it is considered disclosed herein.

141. It is understood that those of skill in the art understand that if a composition or method meets any one of these criteria for determining hybridization either collectively or singly it is a composition or method that is disclosed herein.

### 3. Nucleic acids

142. There are a variety of molecules disclosed herein that are nucleic acid based, including for example the nucleic acids that encode, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2 as well as any other proteins disclosed herein, as well as various functional nucleic acids. The disclosed nucleic acids are made up of for example, nucleotides, nucleotide analogs, or nucleotide substitutes. Non-limiting examples of these and other molecules are discussed herein. It is understood that for example, when a vector is expressed in a cell, that the expressed mRNA will typically be made up of A, C, G, and U. Likewise, it is understood that if, for example, an antisense molecule is introduced into a cell or cell environment through for example exogenous delivery, it is advantageous that the antisense molecule be made up of nucleotide analogs that reduce the degradation of the antisense molecule in the cellular environment.

#### a) Nucleotides and related molecules

143. A nucleotide is a molecule that contains a base moiety, a sugar moiety and a phosphate moiety. Nucleotides can be linked together through their phosphate moieties and sugar moieties creating an internucleoside linkage. The base moiety of a nucleotide can be adenin-9-yl (A), cytosin-1-yl (C), guanin-9-yl (G), uracil-1-yl (U), and thymine-1-yl (T). The sugar moiety of a nucleotide is a ribose or a deoxyribose.

The phosphate moiety of a nucleotide is pentavalent phosphate. An non-limiting example of a nucleotide would be 3'-AMP (3'-adenosine monophosphate) or 5'-γMP (5'-γuanosine monophosphate).

144. A nucleotide analog is a nucleotide which contains some type of  
5 modification to either the base, sugar, or phosphate moieties. Modifications to nucleotides are well known in the art and would include for example, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, and 2-aminoadenine as well as modifications at the sugar or phosphate moieties.

145. Nucleotide substitutes are molecules having similar functional properties  
10 to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

146. It is also possible to link other types of molecules (conjugates) to  
15 nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include but are not limited to lipid moieties such as a cholesterol moiety. (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989,86, 6553-6556),

147. A Watson-Crick interaction is at least one interaction with the Watson-  
20 Crick face of a nucleotide, nucleotide analog, or nucleotide substitute. The Watson-Crick face of a nucleotide, nucleotide analog, or nucleotide substitute includes the C2, N1, and C6 positions of a purine based nucleotide, nucleotide analog, or nucleotide substitute and the C2, N3, C4 positions of a pyrimidine based nucleotide, nucleotide  
25 analog, or nucleotide substitute.

148. A Hoogsteen interaction is the interaction that takes place on the  
Hoogsteen face of a nucleotide or nucleotide analog, which is exposed in the major groove of duplex DNA. The Hoogsteen face includes the N7 position and reactive groups (NH<sub>2</sub> or O) at the C6 position of purine nucleotides.

30 **b) Sequences**

149. There are a variety of sequences related to, for example, RANK, RANKL, TNF-α TNF-R1, and TNF-R2 as well as any other protein disclosed herein

that are disclosed on Genbank, and these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences contained therein.

150. A variety of sequences are provided herein and these and others can be found in Genbank, at [www.pubmed.gov](http://www.pubmed.gov). Those of skill in the art understand how to resolve sequence discrepancies and differences and to adjust the compositions and methods relating to a particular sequence to other related sequences. Primers and/or probes can be designed for any sequence given the information disclosed herein and known in the art.

#### **c) Primers and probes**

151. Disclosed are compositions including primers and probes, which are capable of interacting with the genes disclosed herein. In certain embodiments the primers are used to support DNA amplification reactions. Typically the primers will be capable of being extended in a sequence specific manner. Extension of a primer in a sequence specific manner includes any methods wherein the sequence and/or composition of the nucleic acid molecule to which the primer is hybridized or otherwise associated directs or influences the composition or sequence of the product produced by the extension of the primer. Extension of the primer in a sequence specific manner therefore includes, but is not limited to, PCR, DNA sequencing, DNA extension, DNA polymerization, RNA transcription, or reverse transcription. Techniques and conditions that amplify the primer in a sequence specific manner are preferred. In certain embodiments the primers are used for the DNA amplification reactions, such as PCR or direct sequencing. It is understood that in certain embodiments the primers can also be extended using non-enzymatic techniques, where for example, the nucleotides or oligonucleotides used to extend the primer are modified such that they will chemically react to extend the primer in a sequence specific manner. Typically the disclosed primers hybridize with the nucleic acid or region of the nucleic acid or they hybridize with the complement of the nucleic acid or complement of a region of the nucleic acid.

#### **d) Functional Nucleic Acids**

152. Functional nucleic acids are nucleic acid molecules that have a specific function, such as binding a target molecule or catalyzing a specific reaction. Functional nucleic acid molecules can be divided into the following categories, which are not meant to be limiting. For example, functional nucleic acids include antisense

molecules, aptamers, ribozymes, triplex forming molecules, and external guide sequences. The functional nucleic acid molecules can act as effectors, inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the functional nucleic acid molecules can possess a de novo activity independent of any other molecules.

153. Functional nucleic acid molecules can interact with any macromolecule, such as DNA, RNA, polypeptides, or carbohydrate chains. Thus, functional nucleic acids can interact with the mRNA of RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2 or the genomic DNA of RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2 or they can interact with the polypeptide RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2. Often functional nucleic acids are designed to interact with other nucleic acids based on sequence homology between the target molecule and the functional nucleic acid molecule. In other situations, the specific recognition between the functional nucleic acid molecule and the target molecule is not based on sequence homology between the functional nucleic acid molecule and the target molecule, but rather is based on the formation of tertiary structure that allows specific recognition to take place.

154. Antisense molecules are designed to interact with a target nucleic acid molecule through either canonical or non-canonical base pairing. The interaction of the antisense molecule and the target molecule is designed to promote the destruction of the target molecule through, for example, RNaseH mediated RNA-DNA hybrid degradation. Alternatively the antisense molecule is designed to interrupt a processing function that normally would take place on the target molecule, such as transcription or replication. Antisense molecules can be designed based on the sequence of the target molecule. Numerous methods for optimization of antisense efficiency by finding the most accessible regions of the target molecule exist. Exemplary methods would be in vitro selection experiments and DNA modification studies using DMS and DEPC. It is preferred that antisense molecules bind the target molecule with a dissociation constant ( $k_d$ ) less than or equal to  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-10}$ , or  $10^{-12}$ . A representative sample of methods and techniques which aid in the design and use of antisense molecules can be found in the following non-limiting list of United States patents: 5,135,917, 5,294,533, 5,627,158, 5,641,754, 5,691,317, 5,780,607, 5,786,138, 5,849,903, 5,856,103, 5,919,772, 5,955,590, 5,990,088, 5,994,320, 5,998,602, 6,005,095, 6,007,995,

6,013,522, 6,017,898, 6,018,042, 6,025,198, 6,033,910, 6,040,296, 6,046,004,  
6,046,319, and 6,057,437.

155. Aptamers are molecules that interact with a target molecule, preferably  
in a specific way. Typically aptamers are small nucleic acids ranging from 15-50 bases  
5 in length that fold into defined secondary and tertiary structures, such as stem-loops or  
G-quartets. Aptamers can bind small molecules, such as ATP (United States patent  
5,631,146) and theophiline (United States patent 5,580,737), as well as large molecules,  
such as reverse transcriptase (United States patent 5,786,462) and thrombin (United  
States patent 5,543,293). Aptamers can bind very tightly with  $k_d$ s from the target  
10 molecule of less than  $10^{-12}$  M. It is preferred that the aptamers bind the target molecule  
with a  $k_d$  less than  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-10}$ , or  $10^{-12}$ . Aptamers can bind the target molecule with  
a very high degree of specificity. For example, aptamers have been isolated that have  
greater than a 10000 fold difference in binding affinities between the target molecule  
and another molecule that differ at only a single position on the molecule (United States  
15 patent 5,543,293). It is preferred that the aptamer have a  $k_d$  with the target molecule at  
least 10, 100, 1000, 10,000, or 100,000 fold lower than the  $k_d$  with a background  
binding molecule. It is preferred when doing the comparison for a polypeptide for  
example, that the background molecule be a different polypeptide. For example, when  
determining the specificity of RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2  
20 aptamers, the background protein could be RANK, RANKL, TNF- $\alpha$  TNF-R1, and  
TNF-R2. Representative examples of how to make and use aptamers to bind a variety  
of different target molecules can be found in the following non-limiting list of United  
States patents: 5,476,766, 5,503,978, 5,631,146, 5,731,424, 5,780,228, 5,792,613,  
5,795,721, 5,846,713, 5,858,660, 5,861,254, 5,864,026, 5,869,641, 5,958,691,  
25 6,001,988, 6,011,020, 6,013,443, 6,020,130, 6,028,186, 6,030,776, and 6,051,698.

156. Ribozymes are nucleic acid molecules that are capable of catalyzing a  
chemical reaction, either intramolecularly or intermolecularly. Ribozymes are thus  
catalytic nucleic acid. It is preferred that the ribozymes catalyze intermolecular  
reactions. There are a number of different types of ribozymes that catalyze nuclease or  
30 nucleic acid polymerase type reactions which are based on ribozymes found in natural  
systems, such as hammerhead ribozymes, (for example, but not limited to the following  
United States patents: 5,334,711, 5,436,330, 5,616,466, 5,633,133, 5,646,020,



5,652,094, 5,712,384, 5,770,715, 5,856,463, 5,861,288, 5,891,683, 5,891,684, 5,985,621, 5,989,908, 5,998,193, 5,998,203, WO 9858058 by Ludwig and Sproat, WO 9858057 by Ludwig and Sproat, and WO 9718312 by Ludwig and Sproat) hairpin ribozymes (for example, but not limited to the following United States patents: 5,631,115, 5,646,031, 5,683,902, 5,712,384, 5,856,188, 5,866,701, 5,869,339, and 6,022,962), and tetrahymena ribozymes (for example, but not limited to the following United States patents: 5,595,873 and 5,652,107). There are also a number of ribozymes that are not found in natural systems, but which have been engineered to catalyze specific reactions de novo (for example, but not limited to the following United States patents: 5,580,967, 5,688,670, 5,807,718, and 5,910,408). Preferred ribozymes cleave RNA or DNA substrates, and more preferably cleave RNA substrates. Ribozymes typically cleave nucleic acid substrates through recognition and binding of the target substrate with subsequent cleavage. This recognition is often based mostly on canonical or non-canonical base pair interactions. This property makes ribozymes particularly good candidates for target specific cleavage of nucleic acids because recognition of the target substrate is based on the target substrates sequence. Representative examples of how to make and use ribozymes to catalyze a variety of different reactions can be found in the following non-limiting list of United States patents: 5,646,042, 5,693,535, 5,731,295, 5,811,300, 5,837,855, 5,869,253, 5,877,021, 5,877,022, 5,972,699, 5,972,704, 5,989,906, and 6,017,756.

157. Triplex forming functional nucleic acid molecules are molecules that can interact with either double-stranded or single-stranded nucleic acid. When triplex molecules interact with a target region, a structure called a triplex is formed, in which there are three strands of DNA forming a complex dependant on both Watson-Crick and Hoogsteen base-pairing. Triplex molecules are preferred because they can bind target regions with high affinity and specificity. It is preferred that the triplex forming molecules bind the target molecule with a  $k_d$  less than  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-10}$ , or  $10^{-12}$ . Representative examples of how to make and use triplex forming molecules to bind a variety of different target molecules can be found in the following non-limiting list of United States patents: 5,176,996, 5,645,985, 5,650,316, 5,683,874, 5,693,773, 5,834,185, 5,869,246, 5,874,566, and 5,962,426.

158. External guide sequences (EGSs) are molecules that bind a target nucleic acid molecule forming a complex, and this complex is recognized by RNase P, which cleaves the target molecule. EGSs can be designed to specifically target a RNA molecule of choice. RNase P aids in processing transfer RNA (tRNA) within a cell. Bacterial RNase P can be recruited to cleave virtually any RNA sequence by using an EGS that causes the target RNA:EGS complex to mimic the natural tRNA substrate. (WO 92/03566 by Yale, and Forster and Altman, Science 238:407-409 (1990)).

159. Similarly, eukaryotic EGS/RNase P-directed cleavage of RNA can be utilized to cleave desired targets within eukarotic cells. (Yuan et al., Proc. Natl. Acad. Sci. USA 89:8006-8010 (1992); WO 93/22434 by Yale; WO 95/24489 by Yale; Yuan and Altman, EMBO J 14:159-168 (1995), and Carrara et al., Proc. Natl. Acad. Sci. (USA) 92:2627-2631 (1995)). Representative examples of how to make and use EGS molecules to facilitate cleavage of a variety of different target molecules be found in the following non-limiting list of United States patents: 5,168,053, 5,624,824, 5,683,873, 5,728,521, 5,869,248, and 5,877,162.

#### 4. Nucleic Acid Delivery

160. In the methods described above which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), the disclosed nucleic acids can be in the form of naked DNA or RNA, or the nucleic acids can be in a vector for delivering the nucleic acids to the cells, whereby the antibody-encoding DNA fragment is under the transcriptional regulation of a promoter, as would be well understood by one of ordinary skill in the art. The vector can be a commercially available preparation, such as an adenovirus vector (Quantum Biotechnologies, Inc. (Laval, Quebec, Canada). Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the disclosed nucleic acid or vector can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San

Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

161. As one example, vector delivery can be via a viral system, such as a retroviral vector system which can package a recombinant retroviral genome (see e.g.,  
5 Pastan et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:4486, 1988; Miller et al., *Mol. Cell. Biol.* 6:2895, 1986). The recombinant retrovirus can then be used to infect and thereby deliver to the infected cells nucleic acid encoding a broadly neutralizing antibody (or active fragment thereof). The exact method of introducing the altered nucleic acid into mammalian cells is, of course, not limited to the use of retroviral vectors. Other  
10 techniques are widely available for this procedure including the use of adenoviral vectors (Mitani et al., *Hum. Gene Ther.* 5:941-948, 1994), adeno-associated viral (AAV) vectors (Goodman et al., *Blood* 84:1492-1500, 1994), lentiviral vectors (Naidini et al., *Science* 272:263-267, 1996), pseudotyped retroviral vectors (Agrawal et al., *Exper. Hematol.* 24:738-747, 1996). Physical transduction techniques can also be used,  
15 such as liposome delivery and receptor-mediated and other endocytosis mechanisms (see, for example, Schwartzenberger et al., *Blood* 87:472-478, 1996). This disclosed compositions and methods can be used in conjunction with any of these or other commonly used gene transfer methods.

162. As one example, if the antibody-encoding nucleic acid is delivered to the  
20 cells of a subject in an adenovirus vector, the dosage for administration of adenovirus to humans can range from about  $10^7$  to  $10^9$  plaque forming units (pfu) per injection but can be as high as  $10^{12}$  pfu per injection (Crystal, *Hum. Gene Ther.* 8:985-1001, 1997; Alvarez and Curiel, *Hum. Gene Ther.* 8:597-613, 1997). A subject can receive a single injection, or, if additional injections are necessary, they can be repeated at six month  
25 intervals (or other appropriate time intervals, as determined by the skilled practitioner) for an indefinite period and/or until the efficacy of the treatment has been established.

163. Parenteral administration of the nucleic acid or vector, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension  
30 in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which

is incorporated by reference herein. For additional discussion of suitable formulations and various routes of administration of therapeutic compounds, see, e.g., *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995.

5           164. Nucleic acids that are delivered to cells which are to be integrated into the host cell genome, typically contain integration sequences. These sequences are often viral related sequences, particularly when viral based systems are used. These viral integration systems can also be incorporated into nucleic acids which are to be delivered using a non-nucleic acid based system of deliver, such as a liposome, so that  
10       the nucleic acid contained in the delivery system can be come integrated into the host genome.

          165. Other general techniques for integration into the host genome include, for example, systems designed to promote homologous recombination with the host genome. These systems typically rely on sequence flanking the nucleic acid to be  
15       expressed that has enough homology with a target sequence within the host cell genome that recombination between the vector nucleic acid and the target nucleic acid takes place, causing the delivered nucleic acid to be integrated into the host genome. These systems and the methods necessary to promote homologous recombination are known to those of skill in the art.

20                           **a) In vivo/ex vivo**

          166. As described above, the compositions can be administered in a pharmaceutically acceptable carrier and can be delivered to the subjects cells *in vivo* and/or *ex vivo* by a variety of mechanisms well known in the art (e.g., uptake of naked DNA, liposome fusion, intramuscular injection of DNA via a gene gun, endocytosis and  
25       the like).

          167. If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well known in the art. The compositions can be introduced into the cells via any gene transfer mechanism, such as, for example, calcium phosphate mediated gene delivery, electroporation, microinjection  
30       or proteoliposomes. The transduced cells can then be infused (e.g., in a pharmaceutically acceptable carrier) or homotopically transplanted back into the subject

per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

## 5. Expression systems

168. The nucleic acids that are delivered to cells typically contain expression  
5 controlling systems. For example, the inserted genes in viral and retroviral systems usually contain promoters, and/or enhancers to help control the expression of the desired gene product. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and  
10 transcription factors, and may contain upstream elements and response elements.

### a) Viral Promoters and Enhancers

169. Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses,  
15 hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., Nature, 273: 113 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently obtained  
20 as a HindIII E restriction fragment (Greenway, P.J. et al., Gene 18: 355-360 (1982)). Of course, promoters from the host cell or related species also are useful herein.

170. Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' (Laimins, L. et al., Proc. Natl. Acad. Sci. 78: 993 (1981)) or 3' (Lusky, M.L., et al., Mol. Cell Bio. 3:  
25 1108 (1983)) to the transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., Cell 33: 729 (1983)) as well as within the coding sequence itself (Osborne, T.F., et al., Mol. Cell Bio. 4: 1293 (1984)). They are usually between 10 and 300 bp in length, and they function in cis. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate  
30 the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription. Enhancers often determine the regulation of expression of a gene. While many enhancer sequences are now known from

mammalian genes (globin, elastase, albumin, -fetoprotein and insulin), typically one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples are the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

171. The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

172. In certain embodiments the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. In certain constructs the promoter and/or enhancer region be active in all eukaryotic cell types, even if it is only expressed in a particular type of cell at a particular time. A preferred promoter of this type is the CMV promoter (650 bases). Other preferred promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTF.

173. It has been shown that all specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as melanoma cells. The glial fibrillary acetic protein (GFAP) promoter has been used to selectively express genes in cells of glial origin.

174. Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In certain transcription units, the polyadenylation region is derived from the SV40 early

polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

#### **b) Markers**

5           175. The viral vectors can include nucleic acid sequence encoding a marker product. This marker product is used to determine if the gene has been delivered to the cell and once delivered is being expressed. Preferred marker genes are the *E. Coli lacZ* gene, which encodes  $\beta$ -galactosidase, and green fluorescent protein.

10           176. In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes.  
15           The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR- cells and mouse LTK- cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they  
20           cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

25           177. The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982)), mycophenolic acid, (Mulligan, R.C. and Berg, P. Science 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., Mol. Cell. Biol. 5: 410-413 (1985)). The three examples employ bacterial genes under  
30

eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

## 6. Peptides

### 5 a) Protein variants

178. As discussed herein there are numerous variants of, for example, the RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2 proteins. In addition, to the known functional strain variants there are derivatives of the, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, and TNF-R2 proteins which also function in the disclosed methods  
 10 and compositions. Protein variants and derivatives are well understood to those of skill in the art and in can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple  
 15 amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Immunogenic fusion protein derivatives, such as those described in the examples, are made by fusing a polypeptide sufficiently large to confer immunogenicity to the target sequence by cross-linking in vitro or by recombinant cell culture transformed with  
 20 DNA encoding the fusion. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the  
 25 DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions  
 30 will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final



construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Tables 1 and 2 and are referred to as conservative substitutions.

179. TABLE 2: Amino Acid Abbreviations

<b>Amino Acid</b>	<b>Abbreviations</b>
Alanine	Ala, A
Allosoleucine	AlIle
Arginine	Arg, R
Asparagines	Asn, N
aspartic acid	Asp, D
Cysteine	Cys, C
glutamic acid	Glu, E
Glutamine	Gln, K
Glycine	Gly, G
Histidine	His, H
Isoleucine	Ile, I
Leucine	Leu, L
Lysine	Lys, K
Phenylalanine	Phe, F
Proline	Pro, P
pyroglutamic acidp	Glup
Serine	Ser, S
Threonine	Thr, T
Tyrosine	Tyr, Y
Tryptophan	Trp, W
Valine	Val, V

TABLE 3:Amino Acid Substitutions	
Original Residue Exemplary Conservative Substitutions, others are known in the art.	
Ala	ser
Arg	lys, gln
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn, lys
Glu	asp
Gly	pro
His	asn;gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln;
Met	Leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

180. Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those in Table 3, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the protein properties will be those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine, in this case, (e) by increasing the number of sites for sulfation and/or glycosylation.

181. For example, the replacement of one amino acid residue with another that is biologically and/or chemically similar is known to those skilled in the art as a conservative substitution. For example, a conservative substitution would be replacing one hydrophobic residue for another, or one polar residue for another. The substitutions  
5 include combinations such as, for example, Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. Such conservatively substituted variations of each explicitly disclosed sequence are included within the mosaic polypeptides provided herein.

182. Substitutional or deletional mutagenesis can be employed to insert sites  
10 for N-glycosylation (Asn-X-Thr/Ser) or O-glycosylation (Ser or Thr). Deletions of cysteine or other labile residues also may be desirable. Deletions or substitutions of potential proteolysis sites, e.g. Arg, is accomplished for example by deleting one of the basic residues or substituting one by glutaminy or histidyl residues.

183. Certain post-translational derivatizations are the result of the action of  
15 recombinant host cells on the expressed polypeptide. Glutaminy and asparaginy residues are frequently post-translationally deamidated to the corresponding glutamyl and asparyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of  
20 the o-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco pp 79-86 [1983]), acetylation of the N-terminal amine and, in some instances, amidation of the C-terminal carboxyl.

184. It is understood that one way to define the variants and derivatives of the  
25 disclosed proteins herein is through defining the variants and derivatives in terms of homology/identity to specific known sequences. Specifically disclosed are variants of these and other proteins herein disclosed which have at least, 70% or 75% or 80% or 85% or 90% or 95% homology to the stated sequence. Those of skill in the art readily understand how to determine the homology of two proteins. For example, the  
30 homology can be calculated after aligning the two sequences so that the homology is at its highest level.

185. Another way of calculating homology can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443  
5 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection.

186. The same types of homology can be obtained for nucleic acids by for  
10 example the algorithms disclosed in Zuker, M. *Science* 244:48-52, 1989, Jaeger et al. *Proc. Natl. Acad. Sci. USA* 86:7706-7710, 1989, Jaeger et al. *Methods Enzymol.* 183:281-306, 1989 which are herein incorporated by reference for at least material related to nucleic acid alignment.

187. It is understood that the description of conservative mutations and  
15 homology can be combined together in any combination, such as embodiments that have at least 70% homology to a particular sequence wherein the variants are conservative mutations.

188. As this specification discusses various proteins and protein sequences it is understood that the nucleic acids that can encode those protein sequences are also  
20 disclosed. This would include all degenerate sequences related to a specific protein sequence, i.e. all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while each particular nucleic acid sequence may not be written out herein, it is understood that each and  
25 every sequence is in fact disclosed and described herein through the disclosed protein sequence. It is also understood that while no amino acid sequence indicates what particular DNA sequence encodes that protein within an organism, where particular variants of a disclosed protein are disclosed herein, the known nucleic acid sequence that encodes that protein in the particular organism from which that protein arises is  
30 also known and herein disclosed and described.

189. It is understood that there are numerous amino acid and peptide analogs, which can be incorporated into the disclosed compositions. For example, there are

numerous D amino acids or amino acids, which have a different functional substituent then the amino acids shown in Table 2 and Table 3. The opposite stereo-isomers of naturally occurring peptides are disclosed, as well as the stereo-isomers of peptide analogs. These amino acids can readily be incorporated into polypeptide chains by charging tRNA molecules with the amino acid of choice and engineering genetic constructs that utilize, for example, amber codons, to insert the analog amino acid into a peptide chain in a site specific way (Thorson et al., *Methods in Molec. Biol.* 77:43-73 (1991), Zoller, *Current Opinion in Biotechnology*, 3:348-354 (1992); Ibba, *Biotechnology & Genetic Engineering Reviews* 13:197-216 (1995), Cahill et al., *TIBS*, 14(10):400-403 (1989); Benner, *TIB Tech*, 12:158-163 (1994); Ibba and Hennecke, *Bio/technology*, 12:678-682 (1994) all of which are herein incorporated by reference at least for material related to amino acid analogs).

190. Molecules can be produced that resemble peptides, but which are not connected via a natural peptide linkage. For example, linkages for amino acids or amino acid analogs can include  $\text{CH}_2\text{NH--}$ ,  $\text{--CH}_2\text{S--}$ ,  $\text{--CH}_2\text{--CH}_2\text{--}$ ,  $\text{--CH=CH--}$  (cis and trans),  $\text{--COCH}_2\text{--}$ ,  $\text{--CH(OH)CH}_2\text{--}$ , and  $\text{--CHH}_2\text{SO--}$  (These and others can be found in Spatola, A. F. in *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A. F., *Vega Data* (March 1983), Vol. 1, Issue 3, *Peptide Backbone Modifications* (general review); Morley, *Trends Pharm Sci* (1980) pp. 463-468; Hudson, D. et al., *Int J Pept Prot Res* 14:177-185 (1979) ( $\text{--CH}_2\text{NH--}$ ,  $\text{CH}_2\text{CH}_2\text{--}$ ); Spatola et al. *Life Sci* 38:1243-1249 (1986) ( $\text{--CH H}_2\text{--S}$ ); Hann J. *Chem. Soc Perkin Trans. I* 307-314 (1982) ( $\text{--CH--CH--}$ , cis and trans); Almquist et al. *J. Med. Chem.* 23:1392-1398 (1980) ( $\text{--COCH}_2\text{--}$ ); Jennings-White et al. *Tetrahedron Lett* 23:2533 (1982) ( $\text{--COCH}_2\text{--}$ ); Szelke et al. *European Appln*, EP 45665 CA (1982): 97:39405 (1982) ( $\text{--CH(OH)CH}_2\text{--}$ ); Holladay et al. *Tetrahedron. Lett* 24:4401-4404 (1983) ( $\text{--C(OH)CH}_2\text{--}$ ); and Hruby *Life Sci* 31:189-199 (1982) ( $\text{--CH}_2\text{--S--}$ ); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is  $\text{--CH}_2\text{NH--}$ . It is understood that peptide analogs can have more than one atom between the bond atoms, such as b-alanine, g-aminobutyric acid, and the like.

191. Amino acid analogs and analogs and peptide analogs often have enhanced or desirable properties, such as, more economical production, greater

chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

192. D-amino acids can be used to generate more stable peptides, because D amino acids are not recognized by peptidases and such. Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. Cysteine residues can be used to cyclize or attach two or more peptides together. This can be beneficial to constrain peptides into particular conformations. (Rizo and Gierasch Ann. Rev. Biochem. 61:387 (1992), incorporated herein by reference).

## 7. Antibody

193. As used herein, the term “antibody” encompasses, but is not limited to, whole immunoglobulin (i.e., an intact antibody) of any class. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V(H)) followed by a number of constant domains. Each light chain has a variable domain at one end (V(L)) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains. The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (k) and lambda (l), based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of human immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. One skilled in the art would recognize the comparable classes for mouse.

The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

194. The term “variable” is used herein to describe certain portions of the variable domains that differ in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a b-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the b-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat E. A. et al., “Sequences of Proteins of Immunological Interest,” National Institutes of Health, Bethesda, Md. (1987)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

195. As used herein, the term “antibody or fragments thereof” encompasses chimeric antibodies and hybrid antibodies, with dual or multiple antigen or epitope specificities, and fragments, such as F(ab')<sub>2</sub>, Fab', Fab and the like, including hybrid fragments. Thus, fragments of the antibodies that retain the ability to bind their specific antigens are provided. For example, fragments of antibodies which maintain, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 binding activity are included within the meaning of the term “antibody or fragment thereof.” Such antibodies and fragments can be made by techniques known in the art and can be screened for specificity and activity according to the methods set forth in the Examples and in general methods for producing antibodies and screening antibodies for specificity and activity (See Harlow and Lane. Antibodies, A Laboratory Manual. Cold Spring Harbor Publications, New York, (1988)).

196. Also included within the meaning of “antibody or fragments thereof” are conjugates of antibody fragments and antigen binding proteins (single chain antibodies) as described, for example, in U.S. Pat. No. 4,704,692, the contents of which are hereby incorporated by reference.

5 197. Optionally, the antibodies are generated in other species and “humanized” for administration in humans. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub>, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human  
10 immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by  
15 corresponding non-human residues. Humanized antibodies may also comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or  
20 substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)).

25 198. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as “import” residues, which are typically taken from an “import” variable domain. Humanization can be essentially performed following the method of Winter  
30 and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody.



Accordingly, such “humanized” antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

199. The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the “best-fit” method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993) and Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immunol.*, 151:2623 (1993)).

200. It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and

most substantially involved in influencing antigen binding (see, WO 94/04679, published 3 March 1994).

201. Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J(H)) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge (see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33 (1993)). Human antibodies can also be produced in phage display libraries (Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)). The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol., 147(1):86-95 (1991)).

202. Disclosed are hybridoma cells that produces the monoclonal antibody. The term "monoclonal antibody" as used herein refers to an antibody obtained from a substantially homogeneous population of antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired activity (See, U.S. Pat. No. 4,816,567 and Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)).

203. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975) or Harlow and Lane.

Antibodies, A Laboratory Manual. Cold Spring Harbor Publications, New York, (1988). In a hybridoma method, a mouse or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent.

5 Alternatively, the lymphocytes may be immunized in vitro. Preferably, the immunizing agent comprises, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2.

Traditionally, the generation of monoclonal antibodies has depended on the availability of purified protein or peptides for use as the immunogen. More recently DNA based immunizations have shown promise as a way to elicit strong immune responses and  
10 generate monoclonal antibodies. In this approach, DNA-based immunization can be used, wherein DNA encoding a portion of the, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 expressed as a fusion protein with human IgG1 is injected into the host animal according to methods known in the art (e.g., Kilpatrick KE, et al. Gene gun delivered DNA-based immunizations mediate rapid production of murine monoclonal  
15 antibodies to the Flt-3 receptor. Hybridoma. 1998 Dec;17(6):569-76; Kilpatrick KE et al. High-affinity monoclonal antibodies to PED/PEA-15 generated using 5 microg of DNA. Hybridoma. 2000 Aug;19(4):297-302, which are incorporated herein by referenced in full for the the methods of antibody production) and as described in the examples.

20 204. An alternate approach to immunizations with either purified protein or DNA is to use antigen expressed in baculovirus. The advantages to this system include ease of generation, high levels of expression, and post-translational modifications that are highly similar to those seen in mammalian systems. Use of this system involves expressing domains of the, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2  
25 antibody as fusion proteins. The antigen is produced by inserting a gene fragment in-frame between the signal sequence and the mature protein domain of the, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 antibody nucleotide sequence. This results in the display of the foreign proteins on the surface of the virion. This method allows immunization with whole virus, eliminating the need for purification of target  
30 antigens.

205. Generally, either peripheral blood lymphocytes ("PBLs") are used in methods of producing monoclonal antibodies if cells of human origin are desired, or

spleen cells or lymph node cells are used if non-human mammalian sources are desired.

The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, "Monoclonal Antibodies: Principles and Practice" Academic Press, (1986) pp. 59-103).

5     Immortalized cell lines are usually transformed mammalian cells, including myeloma cells of rodent, bovine, equine, and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme  
10     hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells. Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are  
15     sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection, Rockville, Md. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., "Monoclonal Antibody Production  
20     Techniques and Applications" Marcel Dekker, Inc., New York, (1987) pp. 51-63). The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2. Preferably, the binding specificity of monoclonal  
25     antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art, and are described further in the Examples below or in Harlow and Lane "Antibodies, A Laboratory Manual" Cold Spring Harbor Publications, New York, (1988).

30             206.     After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution or FACS sorting procedures and grown by standard methods. Suitable culture media for this purpose include, for example, Dulbecco's

Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

207. The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, protein G, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

208. The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567. DNA encoding the monoclonal antibodies can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, plasmacytoma cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Optionally, such a non-immunoglobulin polypeptide is substituted for the constant domains of an antibody or substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 and another antigen-combining site having specificity for a different antigen.

209. *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published Dec. 22, 1994, U.S. Pat. No. 4,342,566, and Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York,

(1988). Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields a fragment, called the F(ab')<sub>2</sub> fragment, that has two antigen combining sites and is still capable of cross-linking antigen.

5           210. The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain domain including one or more cysteines from the antibody hinge region. The F(ab')<sub>2</sub> fragment is a bivalent fragment comprising two  
10 Fab' fragments linked by a disulfide bridge at the hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. Antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

15           211. An isolated immunogenically specific paratope or fragment of the antibody is also provided. A specific immunogenic epitope of the antibody can be isolated from the whole antibody by chemical or mechanical disruption of the molecule. The purified fragments thus obtained are tested to determine their immunogenicity and specificity by the methods taught herein. Immunoreactive paratopes of the antibody,  
20 optionally, are synthesized directly. An immunoreactive fragment is defined as an amino acid sequence of at least about two to five consecutive amino acids derived from the antibody amino acid sequence.

          212. One method of producing proteins comprising the antibodies is to link two or more peptides or polypeptides together by protein chemistry techniques. For  
25 example, peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (tert -butyloxycarbonyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the antibody, for example, can be synthesized by standard chemical reactions. For  
30 example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of an antibody can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked

on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) *Synthetic Peptides: A User Guide*. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) *Principles of Peptide Synthesis*. Springer-Verlag Inc., NY. Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described above. Once isolated, these independent peptides or polypeptides may be linked to form an antibody or fragment thereof via similar peptide condensation reactions.

213. For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., *Biochemistry*, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. *Synthesis of Proteins by Native Chemical Ligation*. *Science*, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide-alpha-thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site. Application of this native chemical ligation method to the total synthesis of a protein molecule is illustrated by the preparation of human interleukin 8 (IL-8) (Baggiolini M et al. (1992) *FEBS Lett.* 307:97-101; Clark-Lewis I et al., *J.Biol.Chem.*, 269:16075 (1994); Clark-Lewis I et al., *Biochemistry*, 30:3128 (1991); Rajarathnam K et al., *Biochemistry* 33:6623-30 (1994)).

214. Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. *Science*, 256:221 (1992)). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., *Techniques in Protein Chemistry IV*. Academic Press, New York, pp. 257-267 (1992)).

215. Also disclosed are fragments of antibodies which have bioactivity. The polypeptide fragments can be recombinant proteins obtained by cloning nucleic acids encoding the polypeptide in an expression system capable of producing the polypeptide fragments thereof, such as an adenovirus or baculovirus expression system. For example, one can determine the active domain of an antibody from a specific hybridoma that can cause a biological effect associated with the interaction of the antibody with, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2. For example, amino acids found to not contribute to either the activity or the binding specificity or affinity of the antibody can be deleted without a loss in the respective activity. For example, in various embodiments, amino or carboxy-terminal amino acids are sequentially removed from either the native or the modified non-immunoglobulin molecule or the immunoglobulin molecule and the respective activity assayed in one of many available assays. In another example, a fragment of an antibody comprises a modified antibody wherein at least one amino acid has been substituted for the naturally occurring amino acid at a specific position, and a portion of either amino terminal or carboxy terminal amino acids, or even an internal region of the antibody, has been replaced with a polypeptide fragment or other moiety, such as biotin, which can facilitate in the purification of the modified antibody. For example, a modified antibody can be fused to a maltose binding protein, through either peptide chemistry or cloning the respective nucleic acids encoding the two polypeptide fragments into an expression vector such that the expression of the coding region results in a hybrid polypeptide. The hybrid polypeptide can be affinity purified by passing it over an amylose affinity column, and the modified antibody receptor can then be separated from the maltose binding region by cleaving the hybrid polypeptide with the specific protease factor Xa. (See, for example, New England Biolabs Product Catalog, 1996, pg. 164.). Similar purification procedures are available for isolating hybrid proteins from eukaryotic cells as well.

216. The fragments, whether attached to other sequences or not, include insertions, deletions, substitutions, or other selected modifications of particular regions or specific amino acids residues, provided the activity of the fragment is not significantly altered or impaired compared to the nonmodified antibody or antibody fragment. These modifications can provide for some additional property, such as to



remove or add amino acids capable of disulfide bonding, to increase its bio-longevity, to alter its secretory characteristics, etc. In any case, the fragment must possess a bioactive property, such as binding activity, regulation of binding at the binding domain, etc. Functional or active regions of the antibody may be identified by  
5 mutagenesis of a specific region of the protein, followed by expression and testing of the expressed polypeptide. Such methods are readily apparent to a skilled practitioner in the art and can include site-specific mutagenesis of the nucleic acid encoding the antigen. (Zoller MJ et al. Nucl. Acids Res. 10:6487-500 (1982).

217. A variety of immunoassay formats may be used to select antibodies that  
10 selectively bind with a particular protein, variant, or fragment. For example, solid-phase ELISA immunoassays are routinely used to select antibodies selectively immunoreactive with a protein, protein variant, or fragment thereof. See Harlow and Lane. Antibodies, A Laboratory Manual. Cold Spring Harbor Publications, New York, (1988), for a description of immunoassay formats and conditions that could be used to  
15 determine selective binding. The binding affinity of a monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., Anal. Biochem., 107:220 (1980).

218. Also provided is an antibody reagent kit comprising containers of the monoclonal antibody or fragment thereof and one or more reagents for detecting  
20 binding of the antibody or fragment thereof to, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2. The reagents can include, for example, fluorescent tags, enzymatic tags, or other tags. The reagents can also include secondary or tertiary antibodies or reagents for enzymatic reactions, wherein the enzymatic reactions produce a product that can be visualized.

## 25 8. Pharmaceutical carriers/Delivery of pharmaceutical products

219. As described above, the compositions can also be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector, without causing any  
30 undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient

and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

220. The compositions may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, including topical intranasal administration or administration by inhalant. As used herein, "topical intranasal administration" means delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid or vector. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation. The exact amount of the compositions required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the allergic disorder being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

221. Parenteral administration of the composition, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

222. The materials may be in solution, suspension (for example, incorporated into microparticles, liposomes, or cells). These may be targeted to a particular cell type via antibodies, receptors, or receptor ligands. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Senter, et al., Bioconjugate Chem., 2:447-451, (1991); Bagshawe, K.D., Br. J. Cancer, 60:275-281, (1989); Bagshawe, et al., Br. J. Cancer, 58:700-703, (1988); Senter, et al., Bioconjugate Chem., 4:3-9, (1993); Battelli, et al., Cancer Immunol. Immunother., 35:421-425,

(1992); Pietersz and McKenzie, Immunolog. Reviews, 129:57-80, (1992); and Roffler, et al., Biochem. Pharmacol, 42:2062-2065, (1991)). Vehicles such as "stealth" and other antibody conjugated liposomes (including lipid mediated drug targeting to colonic carcinoma), receptor mediated targeting of DNA through cell specific ligands, lymphocyte directed tumor targeting, and highly specific therapeutic retroviral targeting of murine glioma cells *in vivo*. The following references are examples of the use of this technology to target specific proteins to tumor tissue (Hughes et al., Cancer Research, 49:6214-6220, (1989); and Litzinger and Huang, Biochimica et Biophysica Acta, 1104:179-187, (1992)). In general, receptors are involved in pathways of endocytosis, either constitutive or ligand induced. These receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vesicles, pass through an acidified endosome in which the receptors are sorted, and then either recycle to the cell surface, become stored intracellularly, or are degraded in lysosomes. The internalization pathways serve a variety of functions, such as nutrient uptake, removal of activated proteins, clearance of macromolecules, opportunistic entry of viruses and toxins, dissociation and degradation of ligand, and receptor-level regulation. Many receptors follow more than one intracellular pathway, depending on the cell type, receptor concentration, type of ligand, ligand valency, and ligand concentration. Molecular and cellular mechanisms of receptor-mediated endocytosis has been reviewed (Brown and Greene, DNA and Cell Biology 10:6, 399-409 (1991)).

#### a) Pharmaceutically Acceptable Carriers

223. The compositions, including antibodies, can be used therapeutically in combination with a pharmaceutically acceptable carrier.

224. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy* (19th ed.) ed. A.R. Gennaro, Mack Publishing Company, Easton, PA 1995. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the pharmaceutically-acceptable carrier include, but are not limited to, saline, Ringer's solution and dextrose solution. The pH of the solution is preferably from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of

shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

5           225.    Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of drugs to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositions can be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled  
10   in the art.

          226.    Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

15           227.    The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The disclosed antibodies can be  
20   administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

          228.    Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable  
25   organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the  
30   like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

229. Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

5           230. Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable..

231. Some of the compositions may potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with  
10 inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide,  
15 and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

#### **b) Therapeutic Uses**

232. Effective dosages and schedules for administering the compositions may be determined empirically, and making such determinations is within the skill in the art.

20    The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms disorder are effected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient, route of administration, or  
25 whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products. For example,  
30 guidance in selecting appropriate doses for antibodies can be found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Nokes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et

al., *Antibodies in Human Diagnosis and Therapy*, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

5           233. Following administration of a disclosed composition, such as an antibody, for treating, inhibiting, or preventing a joint inflammation disease the efficacy of the therapeutic antibody can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a composition, such as an antibody, disclosed herein is efficacious in treating or  
10       inhibiting a joint inflammation disease in a subject by observing that the composition reduces viral load or prevents a further increase in the symptoms related to the joint inflammation disease.

          234. There are a variety of ways for determining level of PsA. For example, there is a Psoriatic Arthritis Response Criteria (PsARC). This looks at improvement of  
15       a number of factors at will typically look for improvement of at least 2 of 4 criteria, including, a physician global assessment, a patient global assessment, a tender joint score, and a swollen joint score. A reduction in disease can also be characterized by improvement in at least 1 of 2 joint scores, including no worsening in any criteria and the ACR Response Criteria. ( Clegg DO. *Arthritis Rheum* 1996;39:2013-20). The level  
20       of disease can also be assessed by the psoriasis area and severity index (Fredricksson. *Dermatologica*. 1978) by using the composite index of disease severity which includes evaluation of scale, erythema, induration which can be weighted by severity and by body surface area. The psoriasis area and severity index (PASI; Fredriksson 1978) was used to assess the severity of psoriasis at baseline and any change in severity or lesion  
25       surface area from baseline to the end-of study assessment. In both protocols, only patients with plaque psoriasis involvement  $\geq$  3% body surface area at baseline were evaluated for the PASI. Evaluations also included the percentage of patients achieving a 50%, 75%, and 90% improvement from baseline in PASI score.

## **9. Chips and micro arrays**

30           235. Disclosed are chips where at least one address is the sequences or part of the sequences set forth in any of the nucleic acid sequences disclosed herein. Also

disclosed are chips where at least one address is the sequences or portion of sequences set forth in any of the peptide sequences disclosed herein.

236. Also disclosed are chips where at least one address is a variant of the sequences or part of the sequences set forth in any of the nucleic acid sequences disclosed herein. Also disclosed are chips where at least one address is a variant of the sequences or portion of sequences set forth in any of the peptide sequences disclosed herein.

#### **10. Computer readable mediums**

237. It is understood that the disclosed nucleic acids and proteins can be represented as a sequence consisting of the nucleotides of amino acids. There are a variety of ways to display these sequences, for example the nucleotide guanosine can be represented by G or g. Likewise the amino acid valine can be represented by Val or V. Those of skill in the art understand how to display and express any nucleic acid or protein sequence in any of the variety of ways that exist, each of which is considered herein disclosed. Specifically contemplated herein is the display of these sequences on computer readable mediums, such as, commercially available floppy disks, tapes, chips, hard drives, compact disks, and video disks, or other computer readable mediums. Also disclosed are the binary code representations of the disclosed sequences. Those of skill in the art understand what computer readable mediums. Thus, computer readable mediums on which the nucleic acids or protein sequences are recorded, stored, or saved.

238. Disclosed are computer readable mediums comprising the sequences and information regarding the sequences set forth herein.

#### **11. Compositions identified by screening with disclosed compositions / combinatorial chemistry**

##### **a) Combinatorial chemistry**

239. The disclosed compositions can be used as targets for any combinatorial technique to identify molecules or macromolecular molecules that interact with the disclosed compositions in a desired way. The nucleic acids, peptides, and related molecules disclosed herein can be used as targets for the combinatorial approaches. Also disclosed are the compositions that are identified through combinatorial techniques or screening techniques in which the compositions disclosed in herein, such

as the, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2, or portions thereof, are used as the target in a combinatorial or screening protocol.

240. It is understood that when using the disclosed compositions in combinatorial techniques or screening methods, molecules, such as macromolecular molecules, will be identified that have particular desired properties such as inhibition or stimulation or the target molecule's function. The molecules identified and isolated when using the disclosed compositions, such as, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2, are also disclosed. Thus, the products produced using the combinatorial or screening approaches that involve the disclosed compositions, such as, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2, are also considered herein disclosed.

241. It is understood that the disclosed methods for identifying molecules that inhibit the interactions between, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 can be performed using high through put means. For example, putative inhibitors can be identified using Fluorescence Resonance Energy Transfer (FRET) to quickly identify interactions. The underlying theory of the techniques is that when two molecules are close in space, ie, interacting at a level beyond background, a signal is produced or a signal can be quenched. Then, a variety of experiments can be performed, including, for example, adding in a putative inhibitor. If the inhibitor competes with the interaction between the two signaling molecules, the signals will be removed from each other in space, and this will cause a decrease or an increase in the signal, depending on the type of signal used. This decrease or increasing signal can be correlated to the presence or absence of the putative inhibitor. Any signaling means can be used. For example, disclosed are methods of identifying an inhibitor of the interaction between any two of the disclosed molecules comprising, contacting a first molecule and a second molecule together in the presence of a putative inhibitor, wherein the first molecule or second molecule comprises a fluorescence donor, wherein the first or second molecule, typically the molecule not comprising the donor, comprises a fluorescence acceptor; and measuring Fluorescence Resonance Energy Transfer (FRET), in the presence of the putative inhibitor and the in absence of the putative inhibitor, wherein a decrease in FRET in the presence of the putative inhibitor as compared to FRET measurement in its absence indicates the putative inhibitor



inhibits binding between the two molecules. This type of method can be performed with a cell system as well.

242. Combinatorial chemistry includes but is not limited to all methods for isolating small molecules or macromolecules that are capable of binding either a small molecule or another macromolecule, typically in an iterative process. Proteins, oligonucleotides, and sugars are examples of macromolecules. For example, oligonucleotide molecules with a given function, catalytic or ligand-binding, can be isolated from a complex mixture of random oligonucleotides in what has been referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately  $10^{15}$  individual sequences in 100  $\mu$ g of a 100 nucleotide RNA, to some selection and enrichment process. Through repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in  $10^{10}$  RNA molecules folded in such a way as to bind a small molecule dyes. DNA molecules with such ligand-binding behavior have been isolated as well (Ellington and Szostak, 1992; Bock et al, 1992). Techniques aimed at similar goals exist for small organic molecules, proteins, antibodies and other macromolecules known to those of skill in the art. Screening sets of molecules for a desired activity whether based on small organic libraries, oligonucleotides, or antibodies is broadly referred to as combinatorial chemistry. Combinatorial techniques are particularly suited for defining binding interactions between molecules and for isolating molecules that have a specific binding activity, often called aptamers when the macromolecules are nucleic acids.

243. There are a number of methods for isolating proteins which either have de novo activity or a modified activity. For example, phage display libraries have been used to isolate numerous peptides that interact with a specific target. (See for example, United States Patent No. 6,031,071; 5,824,520; 5,596,079; and 5,565,332 which are herein incorporated by reference at least for their material related to phage display and methods relate to combinatorial chemistry)

244. A preferred method for isolating proteins that have a given function is described by Roberts and Szostak (Roberts R.W. and Szostak J.W. Proc. Natl. Acad. Sci. USA, 94(23)12997-302 (1997). This combinatorial chemistry method couples the

functional power of proteins and the genetic power of nucleic acids. An RNA molecule is generated in which a puromycin molecule is covalently attached to the 3'-end of the RNA molecule. An *in vitro* translation of this modified RNA molecule causes the correct protein, encoded by the RNA to be translated. In addition, because of the attachment of the puromycin, a peptidyl acceptor which cannot be extended, the growing peptide chain is attached to the puromycin which is attached to the RNA. Thus, the protein molecule is attached to the genetic material that encodes it. Normal *in vitro* selection procedures can now be done to isolate functional peptides. Once the selection procedure for peptide function is complete traditional nucleic acid manipulation procedures are performed to amplify the nucleic acid that codes for the selected functional peptides. After amplification of the genetic material, new RNA is transcribed with puromycin at the 3'-end, new peptide is translated and another functional round of selection is performed. Thus, protein selection can be performed in an iterative manner just like nucleic acid selection techniques. The peptide which is translated is controlled by the sequence of the RNA attached to the puromycin. This sequence can be anything from a random sequence engineered for optimum translation (i.e. no stop codons etc.) or it can be a degenerate sequence of a known RNA molecule to look for improved or altered function of a known peptide. The conditions for nucleic acid amplification and *in vitro* translation are well known to those of ordinary skill in the art and are preferably performed as in Roberts and Szostak (Roberts R.W. and Szostak J.W. Proc. Natl. Acad. Sci. USA, 94(23)12997-302 (1997)).

245. Another preferred method for combinatorial methods designed to isolate peptides is described in Cohen et al. (Cohen B.A., et al., Proc. Natl. Acad. Sci. USA 95(24):14272-7 (1998)). This method utilizes and modifies two-hybrid technology. Yeast two-hybrid systems are useful for the detection and analysis of protein:protein interactions. The two-hybrid system, initially described in the yeast *Saccharomyces cerevisiae*, is a powerful molecular genetic technique for identifying new regulatory molecules, specific to the protein of interest (Fields and Song, *Nature* 340:245-6 (1989)). Cohen et al., modified this technology so that novel interactions between synthetic or engineered peptide sequences could be identified which bind a molecule of choice. The benefit of this type of technology is that the selection is done in an intracellular environment. The method utilizes a library of peptide molecules that

attached to an acidic activation domain. A peptide of choice, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 is attached to a DNA binding domain of a transcriptional activation protein, such as Gal 4. By performing the Two-hybrid technique on this type of system, molecules that bind the extracellular portion of, for example, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 can be identified.

246. Using methodology well known to those of skill in the art, in combination with various combinatorial libraries, one can isolate and characterize those small molecules or macromolecules, which bind to or interact with the desired target. The relative binding affinity of these compounds can be compared and optimum compounds identified using competitive binding studies, which are well known to those of skill in the art.

247. Techniques for making combinatorial libraries and screening combinatorial libraries to isolate molecules which bind a desired target are well known to those of skill in the art. Representative techniques and methods can be found in but are not limited to United States patents 5,084,824, 5,288,514, 5,449,754, 5,506,337, 5,539,083, 5,545,568, 5,556,762, 5,565,324, 5,565,332, 5,573,905, 5,618,825, 5,619,680, 5,627,210, 5,646,285, 5,663,046, 5,670,326, 5,677,195, 5,683,899, 5,688,696, 5,688,997, 5,698,685, 5,712,146, 5,721,099, 5,723,598, 5,741,713, 5,792,431, 5,807,683, 5,807,754, 5,821,130, 5,831,014, 5,834,195, 5,834,318, 5,834,588, 5,840,500, 5,847,150, 5,856,107, 5,856,496, 5,859,190, 5,864,010, 5,874,443, 5,877,214, 5,880,972, 5,886,126, 5,886,127, 5,891,737, 5,916,899, 5,919,955, 5,925,527, 5,939,268, 5,942,387, 5,945,070, 5,948,696, 5,958,702, 5,958,792, 5,962,337, 5,965,719, 5,972,719, 5,976,894, 5,980,704, 5,985,356, 5,999,086, 6,001,579, 6,004,617, 6,008,321, 6,017,768, 6,025,371, 6,030,917, 6,040,193, 6,045,671, 6,045,755, 6,060,596, and 6,061,636.

248. Combinatorial libraries can be made from a wide array of molecules using a number of different synthetic techniques. For example, libraries containing fused 2,4-pyrimidinediones (United States patent 6,025,371) dihydrobenzopyrans (United States Patent 6,017,768 and 5,821,130), amide alcohols (United States Patent 5,976,894), hydroxy-amino acid amides (United States Patent 5,972,719) carbohydrates (United States patent 5,965,719), 1,4-benzodiazepin-2,5-diones (United States patent 5,962,337), cyclics (United States patent 5,958,792), biaryl amino acid amides (United

States patent 5,948,696), thiophenes (United States patent 5,942,387), tricyclic  
Tetrahydroquinolines (United States patent 5,925,527), benzofurans (United States  
patent 5,919,955), isoquinolines (United States patent 5,916,899), hydantoin and  
thiohydantoin (United States patent 5,859,190), indoles (United States patent  
5 5,856,496), imidazol-pyrido-indole and imidazol-pyrido-benzothiophenes (United  
States patent 5,856,107) substituted 2-methylene-2, 3-dihydrothiazoles (United States  
patent 5,847,150), quinolines (United States patent 5,840,500), PNA (United States  
patent 5,831,014), containing tags (United States patent 5,721,099), polyketides (United  
States patent 5,712,146), morpholino-subunits (United States patent 5,698,685 and  
10 5,506,337), sulfamides (United States patent 5,618,825), and benzodiazepines (United  
States patent 5,288,514).

249. As used herein combinatorial methods and libraries included traditional  
screening methods and libraries as well as methods and libraries used in iterative  
processes.

15 **b) Computer assisted drug design**

250. The disclosed compositions can be used as targets for any molecular  
modeling technique to identify either the structure of the disclosed compositions or to  
identify potential or actual molecules, such as small molecules, which interact in a  
desired way with the disclosed compositions. The nucleic acids, peptides, and related  
20 molecules disclosed herein can be used as targets in any molecular modeling program  
or approach.

251. It is understood that when using the disclosed compositions in modeling  
techniques, molecules, such as macromolecular molecules, will be identified that have  
particular desired properties such as inhibition or stimulation or the target molecule's  
25 function. The molecules identified and isolated when using the disclosed compositions,  
such as, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 are also disclosed. Thus, the  
products produced using the molecular modeling approaches that involve the disclosed  
compositions, such as, RANK, RANKL, TNF- $\alpha$  TNF-R1, or TNF-R2 are also  
considered herein disclosed.

30 252. Thus, one way to isolate molecules that bind a molecule of choice is  
through rational design. This is achieved through structural information and computer  
modeling. Computer modeling technology allows visualization of the three-

dimensional atomic structure of a selected molecule and the rational design of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

253. Examples of molecular modeling systems are the CHARMM and QUANTA programs, Polygen Corporation, Waltham, MA. CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modeling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

254. A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., 1988 *Acta Pharmaceutica Fennica* 97, 159-166; Ripka, *New Scientist* 54-57 (June 16, 1988); McKinaly and Rossmann, 1989 *Annu. Rev. Pharmacol. Toxicol.* 29, 111-122; Perry and Davies, QSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 *Proc. R. Soc. Lond.* 236, 125-140 and 141-162; and, with respect to a model enzyme for nucleic acid components, Askew, et al., 1989 *J. Am. Chem. Soc.* 111, 1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc., Pasadena, CA., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube, Inc., Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of molecules specifically interacting with specific regions of DNA or RNA, once that region is identified.

255. Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known

compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which alter substrate binding or enzymatic activity.

## 12. Kits

5           256. Disclosed herein are kits that are drawn to reagents that can be used in practicing the methods disclosed herein. The kits can include any reagent or combination of reagent discussed herein or that would be understood to be required or beneficial in the practice of the disclosed methods. For example, the kits could include primers to perform the amplification reactions discussed in certain embodiments of the  
10       methods, as well as the buffers and enzymes required to use the primers as intended.

### D. Methods of making the compositions

257. The compositions disclosed herein and the compositions necessary to perform the disclosed methods can be made using any method known to those of skill in the art for that particular reagent or compound unless otherwise specifically noted.

#### 15           1. Nucleic acid synthesis

258. For example, the nucleic acids, such as, the oligonucleotides to be used as primers can be made using standard chemical synthesis methods or can be produced using enzymatic methods or any other known method. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for  
20       example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Bioscience, Burlington, MA or ABI Model 380B).  
25       Synthetic methods useful for making oligonucleotides are also described by Ikuta *et al.*, *Ann. Rev. Biochem.* **53**:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang *et al.*, *Methods Enzymol.*, **65**:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen *et al.*, *Bioconjug. Chem.* **5**:3-7 (1994).

#### 30           2. Peptide synthesis

259. One method of producing the disclosed proteins is to link two or more peptides or polypeptides together by protein chemistry techniques. For example,

peptides or polypeptides can be chemically synthesized using currently available laboratory equipment using either Fmoc (9-fluorenylmethyloxycarbonyl) or Boc (*tert*-butyloxycarbonyl) chemistry. (Applied Biosystems, Inc., Foster City, CA). One skilled in the art can readily appreciate that a peptide or polypeptide corresponding to the disclosed proteins, for example, can be synthesized by standard chemical reactions. For example, a peptide or polypeptide can be synthesized and not cleaved from its synthesis resin whereas the other fragment of a peptide or protein can be synthesized and subsequently cleaved from the resin, thereby exposing a terminal group which is functionally blocked on the other fragment. By peptide condensation reactions, these two fragments can be covalently joined via a peptide bond at their carboxyl and amino termini, respectively, to form an antibody, or fragment thereof. (Grant GA (1992) *Synthetic Peptides: A User Guide*. W.H. Freeman and Co., N.Y. (1992); Bodansky M and Trost B., Ed. (1993) *Principles of Peptide Synthesis*. Springer-Verlag Inc., NY (which is herein incorporated by reference at least for material related to peptide synthesis). Alternatively, the peptide or polypeptide is independently synthesized *in vivo* as described herein. Once isolated, these independent peptides or polypeptides may be linked to form a peptide or fragment thereof via similar peptide condensation reactions.

260. For example, enzymatic ligation of cloned or synthetic peptide segments allow relatively short peptide fragments to be joined to produce larger peptide fragments, polypeptides or whole protein domains (Abrahmsen L et al., *Biochemistry*, 30:4151 (1991)). Alternatively, native chemical ligation of synthetic peptides can be utilized to synthetically construct large peptides or polypeptides from shorter peptide fragments. This method consists of a two step chemical reaction (Dawson et al. *Synthesis of Proteins by Native Chemical Ligation*. *Science*, 266:776-779 (1994)). The first step is the chemoselective reaction of an unprotected synthetic peptide--thioester with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial covalent product. Without a change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site (Baggiolini M et al. (1992) *FEBS Lett.* 307:97-101; Clark-Lewis I et al., *J.Biol.Chem.*, 269:16075 (1994);

Clark-Lewis I et al., *Biochemistry*, 30:3128 (1991); Rajarathnam K et al., *Biochemistry* 33:6623-30 (1994)).

261. Alternatively, unprotected peptide segments are chemically linked where the bond formed between the peptide segments as a result of the chemical ligation is an unnatural (non-peptide) bond (Schnolzer, M et al. *Science*, 256:221 (1992)). This technique has been used to synthesize analogs of protein domains as well as large amounts of relatively pure proteins with full biological activity (deLisle Milton RC et al., *Techniques in Protein Chemistry IV*. Academic Press, New York, pp. 257-267 (1992)).

## **E. Methods of using the compositions**

### **1. Methods of using the compositions as research tools**

262. The disclosed compositions can be used in a variety of ways as research tools.

263. The compositions can be used for example as targets in combinatorial chemistry protocols or other screening protocols to isolate molecules that possess desired functional properties related to joint inflammation diseases.

264. The disclosed compositions can also be used diagnostic tools related to joint inflammation diseases.

265. The disclosed compositions can be used as discussed herein as either reagents in micro arrays or as reagents to probe or analyze existing microarrays. The disclosed compositions can be used in any known method for isolating or identifying single nucleotide polymorphisms. The compositions can also be used in any known method of screening assays, related to chip/micro arrays. The compositions can also be used in any known way of using the computer readable embodiments of the disclosed compositions, for example, to study relatedness or to perform molecular modeling analysis related to the disclosed compositions.

## **F. Examples**

266. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts,



temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

## 1. Example 1

5

### a) Materials and Methods

#### (1) Study population and treatment protocol

267. All clinical studies were carried out with approval of the University of Rochester Medical Center Research Subjects Review Board and informed consent. Synovium, cartilage and bone specimens were obtained at the time of joint replacement surgery from 5 PsA, 4 RA and 4 OA patients. PsA was diagnosed according to the Moll and Wright criteria (Moll, J.M. and Wright, V. 1973. Psoriatic arthritis. *Seminars in Arthritis & Rheumatism* 3:55-78), RA by the American College of Rheumatology criteria (Arnett, F.C., et al. 1988. *Arthritis Rheum.* 31:315-324) and OA by physical examination and characteristic findings on plain x-ray. A blinded radiologist evaluated radiographs from PsA patients. Healthy controls had no acute or chronic joint pain and were in good health. None of the patients or controls was taking corticosteroids or second line agents (methotrexate, gold, hydroxychloroquine, leflunomide, Enbrel® (etanercept) or infliximab). Nine patients with erosive PsA were treated with anti-TNF agents: 8 patients with Enbrel® (etanercept) 25 mg twice per week and 1 patient with infliximab 5 mg/kg at weeks 0, 2, 6 and 14).

#### (2) Osteoclast precursors from peripheral blood mononuclear cells (PBMCs)

268. PBMCs were isolated from whole blood obtained from 24 PsA patients and 12 healthy controls. The PBMC were separated on Ficoll gradients. Unfractionated PBMC ( $1 \times 10^6$  cells/ml) were placed in 8 well chamber slides containing 0.5ml 10% FCS-RPMI. Cultures were incubated in 6% CO<sub>2</sub> at 37° C for 14 days. Media was replenished every 2-3 days. After 14 days in culture, slides were stained for tartrate resistant acid phosphatase (TRAP) (Sigma Diagnostics St. Louis MO). Slides were viewed by light microscopy and TRAP positive cells with 3 or more nuclei were counted as osteoclasts. The scoring system that was used presents the data as the number of OCP per  $10^6$  PBMC in the initial cell culture even though the final readout is the number of TRAP positive multinuclear cells. This is based on the fact

that osteoclasts are derived exclusively from OCP and there is currently no recognized surface marker for OCP per se. (Massey, H.M. and Flanagan, A.M. 1999. *British Journal of Haematology* 106:167-170; Shalhoub, V., et al. 2000. *Br J of Haematol* 111:501-512)

269. Cultures stimulated with M-CSF (25ng/ml) and RANKL (100ng/ml) served as positive controls. The ability of these cells to resorb bone was demonstrated by culturing PBMC in 0.5 ml 10% FBS-RPMI on bovine bone wafers for 21 days. The cultured bone wafers, together with uncultured wafers, were stained with toluidine blue and photographed. The resorption area was quantified by density scan using Scion imaging software, after subtracting the background in the uncultured wafers. These data are expressed as the % resorption area, calculated by dividing the total pitted area by the total surface area of the bone wafer.

### (3) Flow Cytometry

270. PBMC were prepared as described above and the cells were centrifuged and re-suspended in PBS containing 4% of fetal bovine serum (FBS). Aliquots of  $1 \times 10^6$  cells were incubated with anti-human CD11b (ICRF44), CD14 (M5E2),  $\alpha\nu\beta 3$  (CD 51/61), and related isotype controls (Pharmingen, San Diego, CA), or with fluorescein-conjugated RANKL (a gift from Dr. M. Tondravi, American Red Cross, Rockville, MD). The cells were then washed with 4% FBS-PBS. Data were acquired using a FACScalibur instrument (Beckton Dickinson, Bedford, MA) and analyzed by Cellquest software (ver.3.1).

### (4) Immunohistochemistry on synovial tissues, bone and cartilage

271. All tissue samples were formalin fixed and bone specimens were decalcified in Immunocal (Decal Corporation Congers, NY), dehydrated in a graded series of alcohols and embedded in paraffin. Samples were cut in  $3\mu\text{m}$  sections and mounted on glass slides. Sections were de-paraffinized in xylene and rehydrated through a graded series of alcohols to distilled water. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide. Antigen retrieval was performed in a pressure cooker (de-cloaking chamber, Biocare Medical Walnut Creek, CA) using 0.01M Citrate Buffer. For OPG staining, citrate/glycerol buffer was used. Slides were blocked in 1:20 normal goat serum (Vector Labs Burlingame, Ca). For OPG staining, normal horse serum was used as a blocking agent. Antibodies were diluted as noted below and

incubated overnight at 4°C. Following the incubation, slides were rinsed in PBS and the biotin-conjugated secondary antibodies applied for 30 minutes at room temperature.

Slides were washed and HRP Streptavidin (Zymed Labs, Burlingame, CA) was added at a 1:250 dilution in PBS for 30 minutes at room temperature. Sections were washed

once in PBS followed by deionized water, then incubated in AEC Chromagen

(Romulin, AEC Biocare Medical, Walnut Creek, CA). Slides were counter stained

with hematoxylin. Primary antibodies to RANK (rabbit anti-human ab1861) and RANKL (rabbit anti-human ab1862) were purchased from Chemicon (Temecula, CA).

RANK and RANKL antibodies were diluted in 2% normal goat serum in 1:20

BSA/PBS and applied at a 1:800 dilution. The secondary antibody, biotinylated goat anti-rabbit (Vector Labs, Burlingame CA), was added at 1:200 dilution. OPG antibody

(mouse anti-human MAB805), purchased from R&D Systems (Minneapolis, MN) was used at a dilution of 1:30. The secondary antibody, biotinylated horse anti-mouse

(Vector Labs, Burlingame, CA) was applied at a 1:200 dilution. Sections stained with

only the secondary antibody served as a negative control. Slides were reviewed and

scored by an independent pathologist blinded to the diagnosis. The osteoclast score

was based on an assessment of the number of osteoclasts in Howship's resorption

lacunae per 20 x, intermediate power fields in areas of active bone remodeling. 1+: 1-2

OC per 10 fields (20x), 2+: 2-5 OC per 10 fields (20x), 3+: >5 OC per 10 fields (20x).

#### **(5) Analysis of RANKL, RANK and OPG gene expression by RT-PCR**

272. Synovium was obtained from patients (6 PsA and 2 OA) undergoing total joint replacement or at the time of hand or foot surgery. RNA was isolated as previously described (Ritchlin, C. and Haas-Smith, S.A. 2001. *J of Rheumatol* 28:698-

705), reverse transcribed and PCR carried out under conditions described by Gravallesse Gravallesse, E.M., et al. 2000. *Arthritis Rheum.* 43:250-258) with custom primers from

Gibco Life Technologies (Rockville, MD). Primer sequences: RANKL sense 5' CTATTTTCAGAGCGCAGATGGAT 3' (SEQ ID NO:1); RANKL anti-sense 5' TATGAGAACTTGGGATTTTGATGC 3' (SEQ ID NO:2) (Gravallesse, E.M., et al.

2000. *Arthritis Rheum.* 43:250-258); RANK sense 5' TTAAGCCAGTGCTTCACGGG 3' (SEQ ID NO:3); RANK anti-sense 5' ACGTAGACCACGATGATGTTCGC 3' (SEQ ID NO:4) (Myers, D.E., et al. 1999. *FEBS Letters* 463:295-300); OPG sense 5'

GCTAACCTCACCTTCGAG 3' (SEQ ID NO:5); OPG anti-sense 5'  
 TGATTGGACCTGGTTACC 3' (SEQ ID NO:6)(Huang,L., et al. 2000. *Am J Path*  
 156:761-767); GAPDH sense 5' GCTCTCCAGAACATCATCCCTGCC 3'(SEQ ID  
 NO:7); GAPDH anti-sense 5' CGTTGTCATACCAGGAAATGAGCTT. (SEQ ID  
 5 NO:8)(Gravallese,E.M., et al. 2000. *Arthritis Rheum.* 43:250-258)

#### (6) TNF- $\alpha$ ELISA

273. PBMC cultures were established in 24 well tissue culture plates with  
 cells from 5 PsA patients and 5 healthy controls. Unfractionated PBMCs were plated at  
 1x10<sup>6</sup> cells /ml in 10% FBS-RPMI in 1 ml total volume. The cells were incubated at  
 10 37°C and 6% CO<sub>2</sub> for 14 days. Culture supernatants were harvested and passed through  
 a syringe filter to remove debris. Samples were stored at -20°C until assayed. The  
 assay was performed using matched antibody pairs against human TNF- $\alpha$  (Pierce  
 Endogen Rockford, IL) following the manufactures technical application procedure.  
 Standards were serially diluted recombinant human TNF- $\alpha$  in culture media (Pierce  
 15 Endogen Rockford, IL). Optical density was recorded on a Bio-Rad (Life Technologies  
 Grand Island, NY) microtiter plate reader. TNF- $\alpha$  is expressed as pg/ml.

#### (7) Co-cultures

274. PBMC from PsA patients were cultured in 24 well tissue culture plates  
 at a density of 10 x 10<sup>6</sup> cells /ml in 1.0 ml of 10% FBS RPMI. Supernatants were  
 20 harvested at day 14, filtered and stored at -20°C. Healthy donor PBMC isolated from 3  
 individuals were seeded in 96 well flat bottomed culture plates at 2 x 10<sup>5</sup> cells per well  
 with 50% PsA culture supernatant and 50% normal media in 200  $\mu$ l total volume. In  
 the initial experiments, supernatants from 3 different PsA PBMC cultures were added  
 to PBMC isolated from the 3 healthy controls. In subsequent experiments, supernatant  
 25 from a PsA PBMC culture was added to PBMC isolated from 2 different healthy  
 controls. TNF- $\alpha$  activity was blocked by the addition of anti-TNF- $\alpha$  antibody (Pierce-  
 Endogen Rockford, IL) at a final concentration of 2.5 $\mu$ g/ml. The media was replenished  
 2 times weekly after 14 days in culture; cells were stained for TRAP and osteoclasts  
 were counted as described above.

#### (8) Osteoclastogenesis inhibition by OPG

275. PBMC cultures were established from PsA donors as described above.  
 OPG-Fc (R&D Systems, Minneapolis, MN) was added at a final concentration of 1.0

*ug/ml*). Enbrel® (etanercept) (Amgen, Thousand Oaks, CA) was also added to cultures as indicated at a final concentration of 1 *ug/ml*. Cultures were maintained as described in Methods for 14 days prior to TRAP staining and osteoclast scoring.

5 **(9) Statistics**

276. OCP data are expressed as the number of OCP per 10<sup>6</sup> PBMC. Students t test of non-paired data was used to analyze differences in OCP frequency, resorption area on bone wafers, expression of CD14, CD11b and supernatant TNF- $\alpha$  levels in PsA patients versus healthy controls. The number of PBMC expressing CD14 in PsA patients before and after anti-TNF therapy, OCP numbers before and after supernatant stimulation and blocking experiments with TNF and RANKL were analyzed by paired t tests. The difference in the median number of OCP in PsA patients with and without erosions was analyzed by the Mann-Whitney test.

**b) Results**

15 **(1) Osteoclasts were present in bone obtained from PsA patients**

277. Although it is generally accepted that osteoclasts are the only cell type capable of bone resorption, these cells have not been characterized in the psoriatic joint. To formally document their role in this disease, initial studies were performed to ascertain if osteoclasts were present at sites of focal erosion in PsA bone. Histology specimens from PsA, RA and OA bone were examined and scored for osteoclast number as described in Methods. Histology shows that osteoclasts are prominent in the psoriatic joint. Table 1 shows moderate to large numbers of osteoclasts were detected in bone samples from PsA patients.

**Table 1**

Semiquantitative analysis of osteoclast numbers in PsA, RA, and OA

	Tissue	Osteoclasts
PsA	Hip	3+
	Knee	2+
	Hip	1+
	Foot	1+
	Knee	3+
RA	Hip	2+ to 3+
	Knee	3+
	Foot	2+
	Knee	1+
OA	Knee	0 to 1+
	Hip	0 to 1+
	Knee	0 to 1+
	Knee	0 to 1+

Tissue samples, obtained at surgery, from five patients with PsA, four patients with RA, and four patients with OA were fixed, embedded, sectioned, and stained with H&E. A pathologist blinded to the diagnosis semiquantitatively assessed the number of osteoclasts using the system described in Methods.

278. The majority of osteoclasts were found in resorption pits at the bone-pannus junction, or in cutting cones crossing the subchondral bone. Morphologically mature osteoclasts were not observed in the vascular lumen. Similarly, osteoclasts were increased in RA bone while comparatively few were observed in the OA samples. In some PsA specimens large osteoclasts with high nuclearity (>20 nuclei per cell) were observed in Howship's lacuna.

**(2) Osteoclasts arise in unstimulated cultures of PBMCs from patients with PsA**

279. Numerous multinucleated TRAP positive cells were identified in low density PBMC cultures from PsA patients without exogenous RANKL (100ng/ml) or M-CSF (25ng/ml), while such cells were rare in PBMC cultures from healthy controls. Addition of RANKL and M-CSF to the cultures increased the size and number of osteoclasts in cultures from PsA patients and to a lesser degree in cultures from healthy controls. To quantify this effect, PBMC were isolated from 24 PsA patients and 12 healthy controls (Figure 1). The average number of circulating pre-osteoclasts in

unstimulated cultures was significantly higher in PsA patients compared to healthy controls (mean  $168 \pm 39.9$  vs.  $3.7 \pm 1.1$  OCP per  $10^6$  PBMC;  $p < 0.006$ ). These results indicate that OCP circulate in the peripheral blood of PsA patients in greater numbers than in healthy controls. Furthermore, these precursors progress to mature osteoclasts without exogenous RANKL and M-CSF stimulation.

280. To determine if the increased number of TRAP<sup>+</sup> multinucleated cells are derived from an increase in mononuclear OCP or from multinucleated inflammatory cells, the following experiments were performed. First, PBMCs isolated from 7 PsA patients with erosive arthritis were allowed to settle overnight in 8-well chamber slides.

The cells were fixed, TRAP-stained and examined under a light microscope. Multinucleated or TRAP positive cells in any of the patients were not identified. Second, using probes for surface markers of mononuclear OCP, freshly isolated PBMC from 7 erosive PsA patients and 7 healthy donors were stained for CD11b, CD14, CD51/CD61 and RANK and analyzed by FACS. The percentage of PsA PBMC expressing CD11b and CD14 was significantly greater than in healthy control PBMC ( $23.9\% \pm 3.15\%$  vs.  $13.8\% \pm 1.3\%$ ,  $p < .006$ ). Furthermore, CD11b<sup>+</sup> and CD 14<sup>+</sup> PBMC from PsA patients and controls also expressed CD51/CD61 and RANK, matching a phenotypic profile previously described for OCP (Shalhoub, V., et al. 2000. *Br J of Haematol* 111:501-512). In addition, large or multinucleated cells in the forward and side scatter analysis were not identified.

281. To assess the bone resorbing capacity of these cells, unstimulated PBMC derived from PsA patients and healthy controls were cultured for 21 days on cortical bone wafers. The mean unstimulated values for all patients is shown in Figure 2. Cells from PsA patients (n=6) eroded approximately seven times the surface area as healthy controls (n=6) (mean  $0.49\% \pm 0.31\%$  vs.  $0.08\% \pm 0.12\%$   $p < 0.009$ ). These data demonstrate a functional osteoclast phenotype in cultured PsA PBMC capable of enhanced bone-resorbing activity. This finding is consistent with the increased pre-osteoclast number detected in the PsA population.

### **(3) Circulating pre-osteoclasts are highest in patients with erosive arthritis.**

282. If OCP frequency contributes to inflammatory bone loss in PsA, one would predict that patients with erosions on plain radiographs would have higher

numbers of circulating OPC than PsA patients without erosions. Therefore, OCP frequency was analyzed in unstimulated PBMC cultures from 10 PsA patients with and 10 without bone erosions (Figure 3). PsA patients with one or more erosions on plain radiographs had a significantly greater number of OCP compared to PsA patients without erosions. (median: 224 vs. 85 OCP /10<sup>6</sup> PBMC, p<0.002) These results indicate that OCP contribute to osteolysis in PsA patients.

#### **(4) RANK RANKL and OPG expression in the PsA joint and bone**

283. Based on knowledge of the role of osteoclasts in mediating bone erosions in RA and the importance of RANK-RANKL signaling in the process, the expression pattern of RANK, RANKL and OPG in PsA synovium and bone was investigated. Tissues from PsA patients containing synovium and bone were obtained following surgery and processed for immunohistochemistry as described in Methods. Immunohistochemical analysis revealed that osteoclasts in resorption lacunae strongly express RANK. These RANK positive OC were located at the synovial border of the pannus-bone interface and in cutting cones in the subchondral bone that were void of other cell types. Additionally, RANK positive mononuclear cells increase in number moving from the endothelial cells the erosion front. Furthermore, RANK positive mononuclear cells were detected adjacent to blood vessels traversing the synovium and around vessels located in the subchondral bone. The staining patterns of RANKL and OPG in the synovium was observed. Specifically, retrieval tissues from PsA patients containing synovium were processed for immunohistochemistry with antibodies specific for RANKL and OPG as described in Methods. A representative synovial membrane from a PsA patient stained with anti-RANKL antibody, and anti-OPG antibody, secondary antibody only, and H & E. Intense RANKL immunoreactivity was present throughout the synovial lining layer while OPG staining was restricted to endothelial cells beneath the synovial lining, away from sites of active erosion. Tissue architecture can be determined in H and E stained sections while specific staining is not seen in the negative control. RANK and RANKL staining was weak to absent in all OA tissues examined.

284. To confirm the immunohistochemical studies, RT-PCR was performed on synovial membranes to analyze the pattern of RANK, RANKL and OPG expression



in tissues isolated from 6 patients with PsA and 2 with OA. Briefly, total RNA was isolated from PsA and OA synovium and used as the template to determine RANK, RANKL and OPG mRNA expression by RT-PCR as described in Methods. Five patients with erosive PsA expressed RANKL mRNA in synovial tissues, however, the sixth PsA patient, without bone erosions, did not express RANKL message. The finding of RANK expression in 3 of the 6 PsA synovial samples further supports the immunohistochemical data. Five of 6 PsA and 1 of 2 OA tissues expressed OPG.

#### **(5) Anti-TNF therapy reduces OCP frequency in PsA**

285. It has been previously demonstrated that TNF- $\alpha$  is elevated in psoriatic synovium and synovial fluid (Simonet, W. S., et al. 1997. *Cell* 89:309-319; Ritchlin, C., et al. 1998. *J Rheumatol* 25:1544-1552; Partsch, G., et al. 1998. *Ann. of Rheum. Dis.* 57:691-693). Furthermore, TNF- $\alpha$  can enhance osteoclastogenesis in the presence of miniscule amounts of RANKL (Lam, J., et al. 2000. *J of Clin. Inv.* 106:1481-1488). To delineate the effects of TNF- $\alpha$  on OCP frequency in vivo, five patients with erosive PsA treated with anti-TNF agents (4 with Enbrel® (etanercept) and one with infliximab) were studied. The number of osteoclast precursors was measured before and 12 weeks after initiation of therapy (Figure 4A). Each of the patients experienced a decrease in the number of tender and swollen joints and improved physician and patient global assessment. There was also a significant reduction (79%-96%,  $p < .001$ ) in the number of OCP following anti-TNF therapy in all patients. In addition, a consistent decrease in the percentage of CD11b<sup>+</sup>/CD14<sup>+</sup> PBMCs (9B-D) was observed. These results strongly indicate that TNF- $\alpha$  directly contributes to the increased OCP frequency observed in PsA patients.

#### **(6) Release of biologically active TNF- $\alpha$ by PsA PBMCs**

286. To determine if the increase in OCP is the result of elevated TNF- $\alpha$  in PsA patients, the amount of TNF- $\alpha$  released by unfractionated PBMCs cultured without M-CSF and RANKL from 5 PsA patients and 5 healthy controls was analyzed, (198.6 pg/ml  $\pm$  86.07 vs. 25.8 pg/ml  $\pm$  13.40, respectively ( $p < 0.04$ )). In parallel experiments, PsA culture supernatants harvested from 3 PsA patients with high TNF- $\alpha$  levels stimulated increased osteoclast formation when added in vitro to PBMCs from healthy donors ( $n=3$ ). As shown in Figure 5a unstimulated PBMC from healthy controls yield

few OCP. The number of osteoclasts increased following addition of each of the 3 PsA supernatants. Two of three supernatants significantly increased OCP numbers in the healthy control PBMC cultures. In subsequent experiments, the addition of anti-TNF- $\alpha$  antibody blocked supernatant-induced osteoclastogenesis (Figure 5b). Two healthy controls, different from those in Figure 5a, showed an increase in OCP following addition of PsA supernatant 2 from 5a. This OCP increase was inhibited by addition of anti-TNF antibodies. These experiments indicate that PBMC from PsA patients secrete significantly greater quantities of biologically active TNF- $\alpha$  than PBMC from healthy controls.

#### **(7) OPG inhibits osteoclastogenesis in unstimulated PsA PBMC**

287. Previous studies have convincingly established that RANKL is an essential factor promoting osteoclast development in the inflamed joint (Kong, Y. Y., et al. 1999. *Neurosurgery* 402:304-309; Pettit, A. R., et al. 2001. *Am. J. Pathol.* 159:1689-1699; Redlich, K., et al. 2002. *Arthritis Rheum.* 46:785-792). To examine the impact of RANKL on osteoclast formation in PsA, unstimulated PsA PBMC were cultured in the presence of OPG (Figure 6). The mean number of TRAP positive multinuclear cells in unstimulated cultures was  $45 \pm 5$  per  $10^6$  PBMCs. This number significantly declined to  $14 \pm 4$  in the presence of  $1.0 \mu\text{g/ml}$  of OPG. Since it is known that TNF- $\alpha$  strongly synergizes with trace amounts of RANKL (Lam, J., et al. 2000. *J of Clin. Inv.* 106:1481-1488; Kobayashi K, et al. *J Exp Med* 2000; 191(2):275-86; Azuma Y, et al. *J Biol Chem* 2000; 275(7):4858-64) the combination of OPG and Enbrel® (etanercept) was tested, which further suppressed osteoclast formation to  $8 \pm 2$  OCP per  $10^6$  PBMC. The marked reduction in osteoclast formation in cultures incubated with OPG supports the concept that RANKL expression is a critical event promoting osteoclastogenesis in the psoriatic joint.

288. Taken together with the established literature, the results of this study indicate a mechanism for the destructive pathology observed in many psoriatic joints (Figure 7). In this model, TNF- $\alpha$  increases the number of circulating OCP in PsA patients. In the case of “outside-in” erosion, OCP enter a highly vascular psoriatic synovial membrane containing tortuous blood vessels and adhere to activated endothelial cells that have been stimulated by pro-inflammatory cytokines (Collin-

Osdoby, P., et al. 2001. *J Biol Chem* 276:20659). Exposure to TNF- $\alpha$  can induce the expression of fibronectin and vitronectin receptors on endothelial cells as described by McGowan et al facilitating OCP binding and tissue migration (McGowan, N.W., et al. 2001. *Endocrinology* 142:1678-1681). Simultaneously, the high level of OPG

5 expressed by the endothelial cells would suppress osteoclastogenesis permitting smaller undifferentiated OCP to migrate through the dense pannus and target bone at a significant distance from the vessel. Upon arrival to the bone-pannus junction, OCP bind RANKL on the surface of synoviocytes and in the presence of TNF- $\alpha$  and M-CSF, undergo osteoclastogenesis and erode bone. In the case of “outside-in” resorption, OCP

10 enter the subchondral environment in vessels that are in immediate proximity to bone. Following translocation through the endothelium, it is consistent that OCP are exposed to TNF- $\alpha$  induced RANKL on the surface of osteoblasts and stromal cells (Collin-Osdoby, P., et al. 2001. *J Biol Chem* 276:20659; Hofbauer, L.C., and Heufelder, A.E. 2000. *J Clin Endocrin & Metabolism* 85:2355-2363) resulting in the generation of

15 osteoclasts lining cutting cones devoid of synovial tissue. In this scenario, mature osteoclasts mount a bi-directional assault, resorbing bone matrix in the subchondral bone and at the pannus-bone interface. Thus, there are two critical steps in the osteolytic pathway mediated by TNF- $\alpha$ : increase in the frequency of circulating OCP and upregulation of RANKL expression in the joint. In this model, patients with

20 generalized inflammatory disease (Crohn’s disease, psoriasis) can have an expansion of CD14<sup>+</sup>/CD11b<sup>+</sup> cells that differentiate into dendritic cells or macrophages, but not osteoclasts. In view of the reported findings, antagonism of TNF- $\alpha$  can be an effective strategy for inhibiting bone destruction in PsA.

## 2. Example 2: Systemic TNF $\alpha$ mediates an increase in peripheral CD11b<sup>hi</sup> osteoclast precursors in TNF $\alpha$ transgenic mice

### a) Material and Methods

#### (1) Reagents.

289. Human RANKL and Enbrel® (etanercept) were provided by Dr. W. Dougall (Amgen Inc., Seattle, WA); murine TNF $\alpha$  by Dr. C. R. Dustan (Amgen Inc.,

30 Thousand Oaks, CA) and fluorescein-conjugated RANKL by Dr. M. Tondravi (American Red Cross, Rockville, MD). Recombinant human M-CSF was purchased from R&D Systems Inc. (Minneapolis, MN); anti-murine CD11b (M1/70), c-Fms

(AFS98), CD3 (145-2C11), B220 (RA3-6B2), and isotype controls from eBioscience Inc. (San Diego, CA); anti-murine CD16/32 (FcγIII/II), c-Kit (2B8), Gr-1 (1A8), and isotype controls from Pharmingen (San Diego, CA); anti-murine F4/80 (A3-1) and isotype control from Serotec Ltd. (Oxford, UK).

5 **(2) Animals.**

290. TNF-Tg mice in a CBA x C57Bl/6 background (3647 TNF-Tg line) were obtained from Dr. G. Kollias<sup>5</sup>. The Institutional Animal Care and Use Committee approved all animal studies.

**(3) Generation of osteoclasts.**

10 291. Splenocytes, peripheral blood mononuclear cells (PBMC), and bone marrow cells from TNF-Tg mice and their wild type littermates were used to generate osteoclasts in the absence of osteoblast/stromal cells as described previously (Kobayashi K, et al. *J Exp Med* 2000; 191(2):275-86). These cells were cultured in  $\alpha$ -modified essential medium (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum  
15 (FCS, Hyclone Laboratories, Logan, UT), RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 5 days. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using the Diagnostics Acid Phosphatase Kit (Sigma, St. Louis, MO) to identify osteoclasts. TRAP-positive cells containing  $\geq 3$  nuclei were counted as mature osteoclasts. For the functional study, splenocytes were cultured on bone slices for 10  
20 days under the same conditions as described above. Osteoclasts were then removed and the pits were visualized by 0.1% toluidine blue. The area of pits was quantified, and the data were expressed as the average area of pits ( $\text{mm}^2$ ) /osteoclast  $\pm$  SEM as described previously (Schwarz EM, et al. *Arthritis Res.* 2000; 2:165-168).

**(4) CFU-M colony assay.**

25 292. The *in vitro* colony-forming assay was performed, as described previously (Xing L, et al. *J Bone Miner Res* 2002; 17(7):1200-10). Freshly isolated spleen cells from wt or TNF-Tg mice were plated at a density of  $10^5$  cells/ml in a 35 mm dish. Cells were cultured in the methyl cellulose-based medium (StemCell Technologies Inc, Vancouver, Canada) supplemented with 30 ng/ml of M-CSF for 10  
30 days. Colonies composed of more than 40 cells were counted under an inverted microscope.

**(5) FACS analysis and cell sorting.**

293. Surface protein staining was performed on freshly isolated splenocytes, blood cells, and bone marrow cells. After red blood cell lysis, a single cell suspension was incubated with anti-murine CD16/32 to block Fc receptor-mediated antibody binding. Cells were then labeled with fluorescent probes, as described previously (Li P, et al. *J Immunol* 2000; 164(11):5990-5997). Data were acquired using a FACScalibur instrument (Beckton Dickinson, Bedford, MA) and analyzed by Cellquest software (ver.3.1). Pooled splenocytes from TNF-Tg or wt mice were labeled with anti-murine CD11b or double-stained with anti-murine CD11b and c-Fms antibodies and sorted on a FACScalibur instrument. CD11b<sup>hi</sup>, CD11b<sup>lo</sup>, and CD11b<sup>-</sup> or CD11b<sup>hi</sup>/c-Fms<sup>+</sup> and CD11b<sup>hi</sup>/c-Fms<sup>-</sup> cells were collected separately, reanalyzed to assure their purity (≥98%), and used for osteoclastogenesis assays, as described above.

#### (6) ELISA for human TNFα in mouse serum

294. Blood was drawn from TNF-Tg mice by cardio-puncture, and the serum was collected by centrifugation. The levels of human TNFα were detected according to the manufacturer's instruction (R & D Systems, Minneapolis, MN). The whole procedure was carried at room temperature. Briefly, 96-well plates were coated with 4 μg/ml of capture antibody (MAB610) for overnight and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN<sub>3</sub> for 2 hr. Serum samples and standards were added and incubated for 2 hr. The plates were incubated with 200 ng/ml of biotinylated detection antibody (BAF210) for 1 hr, and then streptavidin HRP (DY 998) for 20 min. The color reaction was developed by adding substrate solutions to the plates, and the OD was read at 450 nm.

#### (7) In vivo BrdU (5-bromo-2-deoxyuridine) labeling.

295. TNF-Tg mice and their wt littermates were given intraperitoneal injections of 1 mg BrdU (Sigma, St. Louis, MO) three times over the course of one day at 8 hr intervals. Spleens were collected 8 hr after the last injection. BrdU staining was performed using the standard immunohistochemistry (Penit C and Vasseur F. *Cytometry* 1993; 14(7):757-63). Briefly, Spleen cell suspensions were surface labeled with anti-CD11b antibody as described above, then fixed and permeabilized in PBS containing 1% paraformaldehyde plus 0.05% Tween 20 for 48-72 hr at 4°C. The cells were treated with 250 U/ml DNase I (Sigma, St. Louis, MO) for 60 min at 37°C, and

BrdU incorporation was revealed with anti-BrdU antibody (Pharmingen, San Diego, CA).

#### **(8) Quantitative real-time PCR.**

296. RNA from TNF $\alpha$  treated and non-treated whole spleen cells, sorted  
 5 CD11b<sup>-</sup>, and CD11b<sup>lo</sup> cells was extracted using the RNeasy kit and the QiaShredder  
 from Qiagen (Valencia, CA). cDNA synthesis was performed, as described previously  
 (Xing L, et al. *J Bone Miner Res* 2002; 17(7):1200-10). Quantitative PCR amplification  
 was performed with gene-specific primers using a Rotor-gene 2000 real time  
 amplification operator (Corbett Research, Mortlake, Australia). The primer sequences  
 10 included: 1) CD11b primers: 5'-ACAGACAAACAGCCCAAACC-3' (SEQ ID NO.  
 24) and 5'-GCCTCACCCATCAGTTGTTT-3', and 2) (SEQ ID NO. 25) actin primers:  
 5'-AGATGTGGATCAGCAAGCAG-3' (SEQ ID NO. 26) and  
 5'-GCGCAAGTTAGGTTTTGTCA-3' (SEQ ID NO. 27). The quantity of CD11b  
 mRNA in each sample was normalized using the CT (threshold cycle) value obtained  
 15 for the actin RNA amplifications run in the same plate.

#### **(9) In vivo blockade with etanercept**

297. TNF-Tg mice (5-month-old, 3 mice per group) were given  
 intraperitoneal injections of Enbrel® (etanercept) (10 mg/kg) or PBS twice a week for 2  
 weeks. The mice were sacrificed 3 days after the last injection, and spleen cells were  
 20 subjected to FACS analysis, osteoclastogenesis, and CFU-M colony assays.

#### **(10) In vivo administration of TNF $\alpha$**

298. Two protocols were used in this study. (1). CBA x C57B/6 mice of 8-  
 week old were first given intraperitoneal injections of BrdU (1mg/mouse), three times  
 daily for three days to obtain a maximal labeling of bone marrow CD11b<sup>+</sup> cells ( $\geq 96\%$ ).  
 25 The mice were then challenged with a single intraperitoneal injection of murine  
 TNF $\alpha$  (1  $\mu$ g/mouse). After 4 hr, bone marrow, spleen, and blood cells were collected  
 for FACS analysis with antibodies to CD11b and BrdU. (2). TNF $\alpha$  (1  $\mu$ g/mouse) was  
 injected into wt mice four times daily for 3 days, as described previously (Boyce BF, et  
 al. *J Clin Invest* 1992; 90(4):1622-7). Two hours after the last injection, spleens were  
 30 taken for FACS analysis, osteoclastogenesis, and CFU-M colony assays. Blood were  
 collected for FACS analysis.

#### **(11) Statistics.**

299. All results are given as means  $\pm$  SEM. Comparisons were made by analysis of variance and Student's *t*-test for unpaired data. P values  $< 0.05$  were considered statistically significant.

5

## b) Results

### (1) Systemic TNF $\alpha$ increases the osteoclastogenic potential of splenocytes and peripheral blood mononuclear cells (PBMC).

300. The TNF-Tg mice (3647 line) used in this study were generated in Dr. Kollias's laboratory (Douni E, et al. *J. Inflam.* 1996; 47:27-38). These mice contain one copy of a modified human *TNF $\alpha$*  transgene in which the ARE-containing 3'UTR was replaced with the 3'UTR from the  $\beta$ -globin gene. This mutation increases the stability and translational efficiency of *TNF $\alpha$*  mRNA and results in chronic TNF $\alpha$  over-expression.

301. To determine if exposure to chronic low levels of TNF $\alpha$  increases osteoclast formation, *in vitro* osteoclastogenesis assays were performed using splenocytes, PBMC, and bone marrow cells from TNF-Tg mice and their wt littermates. Splenocytes and blood cells from TNF-Tg mice cultured with 100 ng/ml of RANKL and 10 ng/ml of M-CSF for 5 days formed more mature osteoclasts than those from wt cells (Fig. 8A). However, no significant differences were observed in the bone marrow cultures. *In vitro* TNF $\alpha$  blockade with a  $10^5$ -fold excess of the TNF $\alpha$  antagonist, Enbrel® (etanercept), over the media concentration of TNF $\alpha$  ( $<10$  pg/ml) had no effect on the enhanced osteoclast formation in TNF-Tg cultures (Fig. 8B). Furthermore, splenocytes from TNF-Tg mice formed 2 to 3-fold more CFU-M colonies than those from wt mice (Fig. 8C). Taken together, these findings indicate that TNF $\alpha$  can stimulate osteoclastogenesis by increasing OCP numbers, but it does not directly affect this process beyond the precursor stage.

302. To examine if TNF $\alpha$  over-expression affects mature osteoclast function *ex vivo*, splenocytes from TNF-Tg and wt mice were cultured on bone slices under the osteoclastogenic condition for 10 days, and measured the area of the resorption pits excavated by the mature osteoclasts. No difference was observed in the resorptive

activity of osteoclasts from the two types of mice (pit area/osteoclast:  $0.0044 \pm 0.0005$  mm<sup>2</sup> in wt vs.  $0.00525 \pm 0.0007$  mm<sup>2</sup> in TNF-Tg mice).

**(2) TNF-Tg mice have increased late stage osteoclast precursors in spleens.**

5           303. Cell surface markers have been used to characterize OCP at various differentiation stages (Arai F, et al. *J Exp Med* 1999; 190(12):1741-54). The earliest OCP, which differentiates from the pluripotent hematopoietic stem cell, is c-Kit<sup>+</sup>/c-Fms<sup>-</sup>/CD11b<sup>-</sup>/RANK<sup>-</sup>. This cell differentiates into the c-Kit<sup>+</sup>/c-Fms<sup>+</sup>/CD11b<sup>-</sup>/RANK<sup>-</sup> early stage precursor and following M-CSF stimulation, proceeds to the c-Kit<sup>-</sup>/c-Fms<sup>+</sup>/CD11b<sup>+</sup>/RANK<sup>+</sup> late stage precursor, which differentiates fully in response to RANKL (Lacey DL, et al. *Cell* 1998; 93(2):165-76; Yasuda H, et al. *Proc Natl Acad Sci U S A* 1998; 95(7):3597-602; Hsu H, et al. *Proc Natl Acad Sci U S A* 1999; 96(7):3540-5; Arai F, et al. *J Exp Med* 1999; 190(12):1741-54). FACS characterization of splenocytes from TNF-Tg mice showed a 4-7 fold increase in the CD11b<sup>+</sup> population compared to wt cells (Fig. 9) and a consistent increase in the c-Fms<sup>+</sup> population. According to their expression levels of CD11b, the CD11b<sup>+</sup> splenocytes can be further divided into CD11b<sup>high</sup> (CD11b<sup>hi</sup>) and CD11b<sup>low</sup> (CD11b<sup>lo</sup>) cells. Only the CD11b<sup>hi</sup> population was significantly increased in TNF-Tg mice compared to that in wt mice (Fig. 10A). To functionally characterize this CD11b<sup>hi</sup> population, CD11b<sup>hi</sup>, CD11b<sup>lo</sup>, and CD11b<sup>-</sup> splenocytes were sorted and cultured them with M-CSF and RANKL. TRAP<sup>+</sup> osteoclasts formed only from the CD11b<sup>hi</sup> population (Fig. 10B, C). Thus, all of OCP capable of forming mature osteoclasts in the culture are in the CD11b<sup>hi</sup> population.

25           304. To further characterize the increased CD11b<sup>hi</sup> cell population, the cells were double stained with antibodies to CD11b and markers for other cell lineages including CD3 (T cells), B220 (B cells), F4/80 (mature macrophages), and Gr-1 (granulocytes). Markers for OCP including c-Kit, c-Fms, and RANK were also investigated. Representative histograms from experiments in which the CD11b<sup>hi</sup> splenocytes were gated on, are shown in Figure 11. Since this population contains both c-Fms<sup>+</sup> and c-Fms<sup>-</sup> cells, the CD11b<sup>hi</sup>/c-Fms<sup>+</sup> and CD11b<sup>hi</sup>/c-Fms<sup>-</sup> sub-populations were sorted and the osteoclastogenesis assay was performed. Both sub-populations had osteoclastogenic potential, and the CD11b<sup>hi</sup>/c-Fms<sup>+</sup> cells formed more osteoclasts than



CD11b<sup>hi</sup>/c-Fms<sup>-</sup> cells (Fig. 12). Thus, CD11b<sup>hi</sup> alone can be used as a representative marker for OCP in the spleen.

305. To determine if there is a correlation between the increased CD11b<sup>hi</sup> OCP frequency and the blood concentration of human TNF $\alpha$  in the transgenic mice, splenocytes were collected and blood from wt and TNF-Tg mice at different ages, corresponding to various stages the development and progression of inflammatory arthritis: prior to onset (1 month), onset (2-3 months), and advanced stage (4 months). The frequency of CD11b<sup>hi</sup> OCP in the spleen was determined by FACS (Fig. 13A), and the concentration of human TNF $\alpha$  in the serum was measured by ELISA (Fig. 13B). Increased CD11b<sup>hi</sup> OCP numbers were first observed in TNF-Tg mice at 2 months of age, corresponding to the time of initial detection of human TNF $\alpha$  in serum and the development of swollen ankles, the first macroscopic sign of inflammatory arthritis. After the onset of arthritis, the frequency of CD11b<sup>hi</sup> OCP and human TNF $\alpha$  serum concentrations remained elevated and did not increase further with progression of erosive arthritis.

**(3) Increased CD11b<sup>hi</sup> osteoclast precursor frequency and enhanced osteoclastogenesis in TNF-Tg mice are reversible by etanercept treatment *in vivo*.**

306. To investigate if TNF $\alpha$  blockade *in vivo* prevents the increases in CD11b<sup>hi</sup> OCP and osteoclast formation, Enbrel® (etanercept) (10 mg/kg) or placebo intraperitoneally was administered into TNF-Tg mice with establish joint disease, twice a week for 2 weeks. Enbrel® (etanercept) reduced the numbers of CD11b<sup>hi</sup> splenocytes (Fig. 14A) and the osteoclastogenic and CFU-M colony-forming potential (Fig. 14B, C) of these cells to wt levels. Thus, the TNF $\alpha$ -mediated increase in OCP is reversible with anti-TNF therapy, which is consistent with the findings of Example 1.

**(4) Systemic TNF $\alpha$  does not affect the proliferation rate, differentiation, or apoptosis of CD11b<sup>hi</sup> osteoclast precursors in the periphery, but can mobilize them from the bone marrow into the circulation.**

307. There are four fundamental mechanisms by which TNF $\alpha$  can increase the number of CD11b<sup>hi</sup> OCP in the periphery: proliferation, survival, differentiation,

and redistribution from the bone marrow. In proliferation assays, TNF-Tg and wt mice were labeled with BrdU for 24 hr, and spleen cells were stained with antibodies against CD11b and BrdU. TNF-Tg mice had the expected increase in CD11b<sup>hi</sup> cells (Fig. 15A), but no increase in the percentage of BrdU<sup>+</sup> CD11b<sup>hi</sup> cells (Fig. 15B, C). In survival  
5 assays, freshly isolated spleen cells from TNF-Tg and wt mice were analyzed by FACS using antibodies against CD11b, fluorescently labeled annexin V, and 7-AAD. In the CD11b<sup>hi</sup> population, the percentage of annexin V<sup>+</sup>/7-AAD<sup>-</sup> cells (apoptotic cells) was similar in TNF-Tg (9.5%) and wt (9.6%) mice. In differentiation assays, wt splenocytes were cultured with TNF $\alpha$  (10 ng/ml), and the percentage of CD11b<sup>hi</sup> OCP after 24 hr  
10 was similar in control and TNF-treated cultures (Fig. 16A), as was the levels of *CD11b* mRNA analyzed by quantitative real-time PCR after 1, 4 and 24 hr TNF treatment (Fig. 16B). Furthermore, *CD11b* mRNA expression assessed by real-time PCR in CD11b<sup>-</sup> and CD11b<sup>lo</sup> splenocytes sorted by FACS, as described in Figure 10, and cultured in the presence of TNF $\alpha$  (10 ng/ml) for 12 hr, was not detectable above background levels  
15 (Fig. 16C).

308. To examine if accumulation of CD11b<sup>hi</sup> cells in the periphery is due to TNF $\alpha$ -induced mobilization of precursors from bone marrow, wt mice were labeled with BrdU for 3 days, then challenged with one injection of TNF $\alpha$  (1 $\mu$ g i.p.) or PBS. After 4 hr, bone marrow, spleen, and blood cells were collected and analyzed by FACS  
20 with antibodies to CD11b and BrdU. No change in the percentage of CD11b<sup>+</sup> cells was observed in bone marrow (Fig. 17A) or spleen, but a 4-fold increase in the percentage of CD11b<sup>+</sup>/BrdU<sup>+</sup> cells in blood (Fig. 17B), indicating that TNF $\alpha$  rapidly mobilizes a small fraction of OCP from the bone marrow to produce a marked increase in the blood. However, further distribution of these cells into peripheral tissues like spleen can  
25 require more time. To test this possibility, wt mice were given TNF $\alpha$  injections for 3 days (1 $\mu$ g/injection, i.p. 4 x per day) and sacrificed 2 hr after the last injection. The percentage of CD11b<sup>hi</sup> splenocytes (Fig. 17C) and PBMC was increased significantly in the TNF $\alpha$ -treated mice compared with controls, which is similar to that observed in untreated TNF-Tg mice. Correspondingly, this treatment caused an increase in the  
30 osteoclastogenic and CFU-M colony-forming potential of the splenocytes from these mice (Fig. 17D).

### c) Discussion

309. A relationship between TNF $\alpha$  and osteoclastic resorption was firmly established in diseases associated with erosive bone loss, such as RA. (Feldmann M, et al. *Cell* 1996; 85(3):307-10; Maini RN, et al. *Immunol Rev* 1995; 144:195-223) TNF $\alpha$  increases osteoclast formation *in vitro* (Abu-Amer Y, et al. *J Biol Chem* 2000; 275(35):27307-10; Kobayashi K, et al. *J Exp Med* 2000; 191(2):275-86; Azuma Y, et al. *J Biol Chem* 2000; 275(7):4858-64), and directly affects OCP *in vivo* in normal mice (Lam J, et al. *J Clin Invest* 2000; 106(12):1481-8). However, in these studies very large amounts of TNF $\alpha$  were used either *in vitro* or *in vivo*, which may not mimic the disease states. Thus, it was important to determine if chronic exposure to a relevant concentration of TNF $\alpha$  affects osteoclast formation, considering the number of RA patients receiving anti-TNF therapy (Moreland LW, et al. *J Rheumatol* 2001; 28(6):1238-44; Keystone EC. *Rheum Dis Clin North Am* 2001; 27(2):427-43) and the potential new indications for other inflammatory bone diseases (Schwarz EM, et al. *Arthritis Res.* 2000; 2:165-168). While it is clear that anti-TNF therapy is efficacious for RA, clinical studies to evaluate its effects on bone resorption have commenced only recently (Lipsky PE, et al. *N Engl J Med* 2000; 343(22):1594-602; Bathon JM, et al. *N Engl J Med* 2000; 343(22):1586-93), and a unifying hypothesis as to how anti-TNF therapy inhibits bone erosion in patients is warranted.

310. In TNF-Tg mice (3647 line), there was a chronic low-level expression of TNF $\alpha$ . As a result, these mice develop an erosive arthritis with features similar to those seen in human RA (Keffer J, et al. *Embo J* 1991; 10(13):4025-31), including focal erosions affecting the immediate subchondral bone and bone at the joint margins. Therefore, this model was appropriate to study the mechanisms of TNF $\alpha$ -mediated osteoclast formation in inflammatory arthritis. As expected, splenocytes from TNF-Tg mice had enhanced osteoclastogenic and CFU-M colony forming potential compared to wt mice (Fig. 8). This enhanced osteoclastogenesis was not inhibited by TNF $\alpha$  blockade *in vitro* (Fig. 8B), but could be recapitulated in cultures of splenocytes from wt mice injected with TNF $\alpha$  (Fig. 17). From these data, it is understood that TNF $\alpha$  had a priming effect on OCP *in vivo* thereby increasing the number of pre-osteoclasts in the periphery outside the bone environment. However, these TNF $\alpha$ -induced pre-osteoclasts did not have increased bone-resorbing capacity, indicating that the systemic

TNF $\alpha$  effect was restricted to increasing the number of these cells and not their function.

311. Systemic TNF $\alpha$  increased the number of pre-osteoclasts in peripheral tissues like spleen and blood. Thus, these cells were identifiable by phenotypic surface markers. Indeed, splenocytes from TNF-Tg mice had 4-7 fold more CD11b<sup>hi</sup> cells  
 5 compared to wt mice. It is important to note that the characterization of the majority of these cells as RANK<sup>-</sup> by FACS (81 % by double staining) did not preclude them as the RANKL responsive pre-osteoclasts that ultimately fuse to form the bone-resorbing cell. These cells could express low levels of functional RANK or up-regulate surface RANK  
 10 expression shortly after stimulation with M-CSF (Arai F, et al. *J Exp Med* 1999; 190(12):1741-54). The CD11b<sup>hi</sup> cells did not express common markers for T or B lymphocytes or mature macrophages, but they did express osteoclast precursor markers such as c-Fms (Fig. 11). Furthermore, all of the OCP in spleen are CD11b<sup>hi</sup> because CD11b<sup>lo</sup> and CD11b<sup>-</sup> cells do not form osteoclasts. Both CD11b<sup>hi</sup>/c-Fms<sup>+</sup> and  
 15 CD11b<sup>hi</sup>/c-Fms<sup>-</sup> populations have the potential to form osteoclasts. Thus, in subsequent studies, CD11b was used as a single surface marker to identify the TNF $\alpha$ -induced pre-osteoclasts.

312. The increase in the number of OPC in the blood was observed between 2 and 3 months of age in the transgenic mice at the same time when blood concentrations  
 20 of TNF $\alpha$  increased. At one month of age, TNF-Tg mice had undetectable blood concentrations of human TNF and normal OPC numbers (Fig. 13). This increase in the number of CD11b<sup>hi</sup> splenocytes and their osteoclastogenic and CFU-M colony forming potential to wt levels was reversible by *in vivo* TNF blockade with Enbrel® (etanercept) (Fig. 14). Based on these two findings, it was concluded that the increase in peripheral  
 25 OCP numbers is due directly to chronic TNF exposure. Consistent with the finding in the TNF-Tg mice, it was demonstrated in Example 1 that patients with psoriatic arthritis (PsA) have a marked increase in the number of pre-osteoclasts in their PBMC population compared to normal and osteoarthritic controls. This increase also appears to be reversible with anti-TNF therapy and can be a dominant mechanism by which this  
 30 treatment inhibits erosions.

313. Last, the cellular mechanisms of TNF $\alpha$ -mediated increase in CD11b<sup>hi</sup> OCP frequency in the periphery was examined. It was observed that accumulation of

these cells was not due to alteration in differentiation, proliferation, or survival of the cells (Figs. 15 and 16), and another mechanism must be involved. In adult life, hematopoietic precursors that give rise to pre-osteoclasts are derived mainly from the differentiation of stem cells in the bone marrow compartment. The finding that injection of TNF $\alpha$  into wt mice induced a remarkable change in the tissue distribution of CD11b<sup>hi</sup> cells, similar to that observed in the blood and spleen of TNF-Tg mice, indicates that redistribution can be a mechanism responsible for the TNF $\alpha$ -mediated increase in peripheral OCP frequency (Fig. 17).

314. Taken together, the findings disclosed herein support a new mechanism for TNF $\alpha$ -induced erosive arthritis in which a significant component of its effect could be due to the mobilization of CD11b<sup>hi</sup> OCP from the bone marrow, thereby increasing their numbers in the circulation. The results of these studies show that the frequency of CD11b<sup>hi</sup> PBMC could be used as a diagnostic to identify patients with active disease or “flares” that can lead to further bone erosion. Furthermore, patients that are responsive or refractory to anti-TNF therapy can be identified by changes in this population following therapy.

### 3. Example 3

#### a) Materials and Methods

315. The independent variable was patients with erosive arthritis on Enbrel® (etanercept). The two primary response variables were the change in the number of circulating OCP and change in level of enhancing bone marrow edema (EBME) on MRI in one inflamed joint after 6 months of Enbrel® (etanercept) treatment.

#### (1) Patient Enrollment and Assessment

316. Twenty PsA patients (Moll & Wright criteria) over the age of 18 with >1 erosions on plain radiographs were given Enbrel® (etanercept) 25 mg twice per week in an open label trial for 6 months. Patients were excluded if they were taking a DMARD, an anti-TNF agent or had previously taken an anti-TNF agent. The total joint count, total number of swollen joints, physician and patient global assessment and Health Assessment Questionnaire (HAQ) scores were recorded at baseline, 2 week, 3 month and 6 month intervals.

## (2) Conventional and 3 Dimensional MRIs

317. Gadolinium (Gd) enhanced and 3 dimensional (3D) MRIs were performed at baseline and at 6 months in all 20 patients. 6 wrists, 3 thumbs, 3 knees and 1 toe were imaged. T2W fat suppressed Fast Spin Echo (2-3 mm slices) were acquired in sagittal plane. The fat suppressed T1W pre and post Gadolinium SE images were acquired in axial plane. Two radiologists independently scored enhancing bone marrow edema (EBME) lesions using a 9 point scale measuring intensity of EBME on T2-weighted imaging (0-3), contrast enhancement post-gd (0-3) and size of lesion (0-3).

## (3) Level of Circulating OCP

318. The frequency of OCP was measured at baseline, 2, 12 and 14 weeks. OCP levels were obtained by counting the number of tartrate resistant acid phosphatase (TRAP) positive cells with > 3 nuclei present in unstimulated peripheral blood mononuclear cells (PBMC) after 2 weeks in culture.

### b) Results

319. All patients completed the trial and no adverse events were reported. MRI data on 13 patients have been analyzed to date (Table 4). Patients experienced decreased tender (24 to 9,  $p < .001$ ) and swollen (27 to 10,  $p < .001$ ) joints and improved physician and patient global assessments (3.5 to .68,  $p < .001$ , 3.5 to 1.3,  $p < .001$ ) after 6 months of therapy (Table 5). A significant decline in the HAQ and SF36 was also noted. OCP were elevated in 18 of 20 patients at baseline ranging from 4 to 226 OC/106 PBMC. Overall, OCP decreased from a mean of  $55.45 \pm 73.4$  to  $11.4 \pm 13.8$  OC/106 PBMC, ( $p < .01$ ) at 6 months. A significant drop in OCP was noted as early as 2 weeks in 14 of 20 patients after starting Enbrel® (etanercept) therapy ( $p < .04$ ). Mean EBME decreased from 5.9 to 3.9, ( $p < .004$ ). Interestingly, in 10 of the 13 patients who had MRIs, a decrease in OCP was associated with lessening EBME. In one patient with no change in OCP at 6 months, EBME increased (Fig. 18 and 19).

Table 4.

Demographics:	
number of subjects	20
male/ female	13 / 7
mean age	47.5 years

mean duration of Ps	21.2 years
mean duration of PsA	11.8 years
previous DMARDs	13 / 20

Table 5.

Outcome measures	Screening	6 Months	p value
Number of Tender joints	24	9	0.001
Number of Swollen Joints	27	10	0.001
MD global assessment	3.5	0.68	0.001
Patient assessment	3.5	1.3	0.001
HAQ score	1.21	0.36	0.0001
SF-36: physical component	31.17	46.30	0.0001
SF-36: mental component	46.07	53.94	0.017

**c) Conclusion:**

5           320.    PsA patients taking Enbrel® (etanercept) demonstrated significant improvement in all clinical parameters as early as 2 weeks after beginning therapy with continued improvement noted over the next 22 weeks.

          321.    Increased OCP were noted at baseline in almost all patients and the frequency of OCP declined significantly after Enbrel® (etanercept) therapy. The  
10   decline in OCP frequency was associated with a significant decline in EBME on conventional and 3D MRI. Persistent EBME was present after 6 months of Enbrel® (etanercept) therapy in many patients despite significant improvement in clinical symptoms and decline in OCP frequency

          322.    PsA patients on Enbrel® (etanercept) demonstrated significant  
15   improvement in all clinical parameters at 6 months. Increased OCP were observed in the majority of PsA pts. Moreover, the decline in OCP frequency was associated with lessening EBME in individual pts. These findings coupled with the rapid decline in OCP following treatment suggest a potential mechanism to explain the protective effects of Enbrel® (etanercept) on inflammatory bone loss in PsA.

#### 4. Example 4

##### a) CD11b<sup>+</sup> osteoclast precursors (OCP) frequency as a marker of erosive disease.

323. In an effort to understand the mechanism of TNF $\alpha$ -induced  
5 osteoclastogenesis in the hTNF-Tg mice, and to develop a quantitative hematological  
outcome measure of erosive disease, the OCP frequency in the spleen and blood (Figure  
22) was determined. It was found that the OCP frequency in the spleen and PBMC of  
hTNF-Tg mice can be markedly increased compared to non-transgenic controls as  
determined by osteoclastogenesis and the number of colonies that form on  
10 methylcellulose media supplemented with M-CSF (CFU-M colonies) (Figure 22A).  
However, osteoclasts from TNF-Tg mice did not demonstrate a significant increase in  
activity as determined by *in vitro* bone wafer resorption assays. In order to better  
characterize the TNF-induced OCP, an extensive FACS analysis with various  
antibodies against hematopoietic surface markers was performed. Of these, CD11b  
15 proved to be the most useful, as cell sorting experiments showed that all of the OCP are  
in the CD11b<sup>hi</sup> population and that TNF-Tg mice have a significant increase in this  
population.

324. In the next series of experiments, it was established that anti-TNF  
therapy completely reverses this phenotype in TNF-Tg mice (Figure 23A-C). This was  
20 done by treating the TNF-Tg mice with Enbrel® (etanercept) for 2-weeks and assaying  
for CD11b, TRAP osteoclastogenesis and CFU-M colony forming units. Furthermore,  
it was demonstrated that TNF-induced OCP frequency could be recapitulated in wild-  
type mice injected with TNF (Figure 23D-F). These results indicate that systemic TNF  
can induce and increase OCP frequency in peripheral tissues. The OCP response can be  
25 transient and can require continuous TNF stimulation to be maintained.

325. An analysis of serum TNF levels vs. OCP frequency vs. inflammatory-  
erosive arthritis was also performed. These studies demonstrated a direct correlation  
between these variables. In the 3647 TNF-Tg mice, synovitis, focal erosions, increased  
serum TNF levels and increased OCP frequency all commence at two-months of age.



**b) TNF induces CD11b<sup>+</sup> OCP frequency independent of RANK signaling.**

326. As stated above, it has become clear that RANK signaling can be required for osteoclastic bone resorption *in vivo*. In order to better characterize the TNF-induced OCP and determine if RANK signaling is required for their formation, hTNF-Tg mice were crossed with RANK knockout mice (hTNF-Tg x RANK<sup>-/-</sup>) (Figure 24). To assess the effects of the cross, bone density and tooth eruption were assessed by x-ray radiography. It was found that the phenotype of the double transgenics was essentially identical to that of their RANK<sup>-/-</sup> littermates, in that the animals had no osteoclasts, failed tooth eruption and suffer from severe osteopetrosis. Additionally, tibia from WT, TNF-Tg, RANK<sup>-/-</sup>, and TNF-Tg x RANK<sup>-/-</sup> mice were prepared for histology, stained for TRAP activity, and counter-stained with hematoxylin/fast-green. The only difference was the presence of some inflammation in some joints, which was markedly reduced from that observed in the hTNF-Tg littermates. Moreover, the extramedullary hematopoiesis combined with the systemic TNF in these animals generated a very large population of CD11b<sup>+</sup> cells in spleen, demonstrating that RANK signaling is not required for this event.

327. In a second model, the effects of RANK:Fc (10mg/kg/48hr) were evaluated in 4-month-old hTNF-Tg mice with established arthritis and found that RANK blockade rapidly eliminates osteoclasts at the leading edge of the erosion front and at sites of normal bone remodeling, but has no effect on OCP frequency (Figure 25). This information was obtained by staining for TRAP activity and counter staining with hematoxylin/fast-green and then comparing photomicrographs of sections from PBS and RANK:Fc treated hTNF-Tg mice. Osteoclast numbers in the tibia (remodeling) and at the pannus-bone junction (erosion) of the hTNF-Tg mice were quantified by histomorphometry. A similar analysis was performed with H&E and TRAP stained sections to evaluate bone erosion, which was done by dividing the eroded surface in mm by the total surface of the proximal tibia and distal femur. These results confirm that RANK signaling is not required for the generation of TNF-induced OCP, but can be required for the differentiation of these cells into active osteoclasts *in vivo*.

**c) TNF-induced CD11b+ OCP in PsA correlates with erosive disease.**

328. Based on studies of TNF-induced OCP in mice described above, clinical studies were commenced to see if these observations would hold true in humans. A study to evaluate OCP was performed in a subset of these patients with and without erosive arthritis before and after anti-TNF therapy. These studies revealed several remarkable findings including: 1) expression of RANK, RANKL and OPG in the PsA joint, 2) OCP frequency in PBMC of PsA patients is significantly higher than healthy controls 3) the OCP frequency in PBMC of PsA patients with erosions can be significantly greater than that in PsA patients without erosions (Figure 26A), and that 12-weeks of anti-TNF therapy significantly reduced the OCP frequency in 5 of 5 patients tested (Figure 26B-D). In addition to the standard clinical outcome measures (Mease, P. J. 2002. *Ann Rheum Dis* 61:298), OCP frequency in PBMC was also evaluated. These data confirmed that PsA patients have a significant increase in OCP frequency that can be reversible with anti-TNF therapy. To the end of quantifying the effects of this treatment on erosive disease in these patients, bone marrow edema was evaluated, which has been validated as a predictive marker by Gd-enhanced fat suppressed MRI. This technique was modified with VirtualScopics technology to generate precision volumetric measurements as was done for periacetabular osteolysis. Figure 18 shows the quantitative radiology results from the first 13 patients and reconstructed 3D-MRI images from a representative patient. These preliminary data formally demonstrate the feasibility of the approach and provide the first evidence in humans that there is a direct correlation between TNF-induced OCP frequency and bone marrow edema in erosive disease; and that anti-TNF therapy corrects both abnormalities.

**d) Biased monocyte differentiation towards osteoclastogenesis has an inverse effect on macrophage and dendritic cell potential.**

329. Disclosed herein, aberrant myelopoiesis and monocyte effector function induces an increase in OCP and erosive disease. In an attempt to validate the working hypothesis it was demonstrated the reciprocal inhibitory effects of IFN- $\gamma$  on osteoclastogenesis and RANKL suppression of macrophage activation (Huang, W., et

al. 2003. *Arthritis Res Ther* 5:R49). In these studies it was shown that IFN- $\gamma$  stimulation of OCP leads to the immediate and complete loss of their osteoclastic potential. Furthermore, a mutiparameter assays was utilized to demonstrate that co-

5 However, if these OCP were treated with RANKL 48hr before IFN- $\gamma$  stimulation, the inhibitory effects were not observed. By assaying these same cultures for NO production and expression of IFN- $\gamma$  inducible surface markers, it was also discovered that RANKL pretreatment (osteoclastogenesis) leads to the loss of macrophage activation potential (Figure 27).

10 330. Very recently, the first evidence that the increase in PBMC OCP frequency in PsA occurs with a concomitant decrease in DCP frequency was obtained. Figure 28 shows the results of an experiment in which purified CD11c+ monocytes from a naïve PsA patient and an age/sex matched healthy control were analyzed for their ability to differentiate in response to GM-CSF and IL-4. These individuals had an

15 equivalent distribution of CD11c+ monocytes in their blood that displayed a similar induction of the IL-4 inducible marker CD209 after 6 days of culture. However, the PsA cells demonstrated a remarkable defect in the induction of the mature dendritic cell marker CD1a+ compared to the control (57.6% vs 100.1% conversion).

20 331. Delneste et al demonstrated that addition of IFN $\gamma$  to IL-4 plus GM-CSF-stimulated monocytes switches their differentiation from CD1a+ DCs to CD14(-)CD64(+) macrophages (Delneste, Y., et al. 2003. *Blood*. 101:143). Results from neutralizing experiments showed that both M-CSF and IL-6 are involved in the ability of IFN $\gamma$  to skew monocyte differentiation from DCs to macrophages. Similar to the findings with OCP, these investigators found that the IFN $\gamma$  effect can be limited to early

25 stages of differentiation. When added to immature DCs, IFN $\gamma$  does not convert them to macrophages, even in the presence of exogenous M-CSF and IL-6. This interplay between IL-6 and M-CSF that switches monocyte differentiation to macrophages rather than DCs was also demonstrated by Chomarat et al (Chomarat, P., et al. 2000. *Nat Immunol* 1:510), suggesting that STAT signaling can be the central pathway through

30 with both OCP and DCP are removed from their respective lineages during monocyte differentiation towards an activated macrophage.

332. Disclosed herein a unifying model is proposed to explain the mechanism through which chronic innate autoimmunity dictates the erosive vs nonerosive phenotype in inflammatory arthritis (Figure 29). This model posits: 1) osteoclasts are responsible for bone erosions; 2) osteoclasts are derived from CD11b+/CD14+ myeloid precursors that are released from bone marrow into the blood. Macrophages and myeloid dendritic cells are also derived from this CD11b+/CD14+ myeloid precursors; 3) TNF directly and/or indirectly stimulates the RANKL + M-CSF mediated differentiation of these cells into a mononuclear OCP that loses its potential to transdifferentiate into activated macrophages or dendritic cells; 4) a mutually exclusive monocyte differentiation cascade also exists for macrophage activation and DC differentiation, which can be mediated by distinct signals, resulting in a loss of osteoclastic potential; and 5) the systemic IFN signal in SLE stimulates DC differentiation at the expense of osteoclastogenesis resulting in a nonerosive phenotype.

**e) Determine if a divergent pathway of monocyte differentiation is the central mechanism underlying the erosive phenotype in inflammatory arthritis.**

333. Disregulated innate immunity in inflammatory arthritis leads to systemic TNF that directly and indirectly through M-CSF and RANKL biases monocyte differentiation towards osteoclastogenesis and away from the macrophage and dendritic cell lineages. This results in an increase in PBMC OCP frequency and a decrease in DCP and macrophage activation potential. These phenomena are reversible via anti-TNF therapy and are absent in patients with SLE because systemic IFN $\alpha$  biases monocyte differentiation toward dendritic cells and away from osteoclasts. Thus, the TNF/IFN axis in inflammatory arthritis dictates erosive disease via these mechanisms.

334. In clinical trials it was demonstrated that PsA patients have a significant increase in CD11b+ OCP in their PBMC that correlates with erosive disease and can be reversible with anti-TNF therapy. It was also demonstrated that a large component of SLE can be IFN $\alpha$ -induced monocyte differentiation to the dendritic cell lineage (Blanco, P., et al. 2001. *Science* 294:1540). Since it is known that inflammatory arthritis in SLE is rarely erosive and that osteoclasts and dendritic cells are derived from the same myeloid precursor, it is shown that these processes are mutually exclusive.

**(1) Experimental Design and Methods:**

335. The OCP frequency, DCP and macrophage activation potential are determined in peripheral blood mononuclear cells (PBMC) from 20 normal healthy controls, 20 erosive PsA patients, 20 SLE patients with non-erosive arthritis and 20 SLE patients erosive arthritis. Sharp Scores can be determined from x-rays performed  
 5 in SLE and PsA patients. Serum TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 levels can be determined as well, and total RNA samples from the PBMC can be banked.

336. The impact of TNF-blockade therapy on OCP and DC and macrophage activation in PBMC can be determined in the 20 erosive PsA patients 6 months after  
 10 treatment with Enbrel® (etanercept), infliximab or adalimumab. Sharp Scores can be determined from x-rays. Serum TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 levels can be determined as well, and total RNA samples from the PBMC can be banked. Additionally, the erosive phenotype in each patient by MRI before and 6 months after therapy can be determined. Clinical assessment, plain radiographs and  
 15 cellular and serologic studies can be repeated after 6 months of therapy with Enbrel® (etanercept), adalimumab or infliximab.

***(a) SLE Patients.***

337. Twenty SLE patients with nonerosive arthritis, and twenty SLE patients with erosive arthritis are recruited SLE patients with inflammatory arthritis, defined as  
 20 joint swelling and pain with morning stiffness in the absence of clinical features of osteoarthritis, are evaluated. Plain radiographs of hands and feet are performed, and patients can be split into 2 groups based on the presence or absence of erosive arthritis.

***(b) Healthy Normal Controls.***

338. Healthy controls are recruited from the general population and receive a  
 25 standard honorarium for their blood donation.

***(c) Evaluation of the erosive phenotype.***

339. Plain x-ray films are obtained from the PsA patients at baseline and 6-months after anti-TNF therapy. Plain x-rays films are also obtained from the SLE patients with inflammatory arthritis.

***(d) Purification of monocytes from PBMC.***

340. 30ml of EDTA anticoagulated blood from each individual can be  
 30 processed with Rosettsept and a Ficoll Paque Plus separation according to the

manufacture's instructions (StemCell Technologies Inc). The cells are then resuspended in PBS and counted. This process typically yields ~10-12 million monocytes that contains >2% lymphocytes.  $10^6$  of these cells can be used immediately for OCP and DCP analysis by FACS.  $2 \times 10^6$  of these cells can be used for TRAP osteoclastogenesis.  $10^6$  of these cells can be used for CFU-M colony formation.  $10^6$  of these cells can be used for NO assays.  $2 \times 10^6$  of these cells can be used to assay macrophage activation by FACS.  $2 \times 10^6$  of these cells can be used to assay for dendritic cell differentiation by FACS.

***(e) OCP Frequency and Osteoclastogenesis.***

341. OCP and DCP frequency can be determined simultaneously, immediately after monocyte purification by four-color FACS.  $10^6$  monocytes are separated into two tubes. The cells in the first tube are stained with CD11b-PE (clone ICRF44), CD11c-APC (clone B-ly6), CD64-FITC (clone 10.1) and CD33-PerCP-Cy5.5 (clone P67.6) antibodies (BD Pharmingen). The cells in the other tube are stained with isotype controls (BD Pharmingen). These samples are then run through a FACScalibur instrument (Beckton Dickinson) and analyzed with Cellquest software (ver.3.1) as done previously. The OCP frequency can be determined by the % of CD11b stained cells that have a MFI greater than 99% of the PE-conjugated isotype control stained cells. TRAP osteoclastogenesis can be performed as described previously. Briefly,  $2 \times 10^6$  monocytes are divided into 4 wells on a glass culture slide (Costar) and cultured in  $\alpha$ -MEM (GIBCO BRL) supplemented with 10% FCS (Hyclone Laboratories), M-CSF (25ng/ml) and RANKL (100ng/ml) (R&D Systems) and incubated in 6% CO<sub>2</sub> at 37° C for 14 days. Media can be replenished every 3 days. After 14 days in culture, the slides are stained for TRAP (Sigma Diagnostics) and the number of TRAP positive cells with 3 or more nuclei (osteoclasts) per  $10^6$  monocytes plated can be determined by light microscopy.

***(f) CFU-M Colony Formation and Macrophage Activation.***

342. The *in vitro* colony-forming assay can be performed as described herein. Briefly,  $2.5 \times 10^5$  freshly isolated monocytes are plated in 2.5ml of methyl cellulose-based medium (StemCell Technologies Inc) supplemented with 30 ng/ml of M-CSF in 35mm culture dishes for 10 days (N=4). Colonies composed of more than 40 cells are

counted under an inverted microscope and the data are presented as CFU-M colonies per  $10^6$  monocytes plated. Macrophage activation can be determined by NO respiratory burst assays and FACS analysis as described previously (Huang, W., et al. 2003.

*Arthritis Res Ther* 5:R49). NO production can be measured by the Greiss reaction

5 (Promega), which spectrophotometrically detects nitrite, a stable breakdown product of nitric oxide whose accumulation reflects NO production. Briefly,  $10^4$  freshly isolated monocytes are suspended in 200 $\mu$ l of  $\alpha$ -MEM (GIBCO BRL) supplemented with 10% FCS (Hyclone Laboratories), plated in 96-well plates (N=4) and left untreated (control) or activated with 1 $\mu$ g/ml LPS (Sigma), and/or 10ng/ml IFN- $\gamma$  (R&D Systems). Cell  
10 supernatants are harvested at 24hr and reacted with 1% sulfanilamide and 0.1% naphthyl-ethylenediamine. A standard curve can be constructed using dilutions of sodium nitrite, and absorbance can be measured at 550nm. Total protein concentration in each well can be used to normalize the data to control for variation in cell number or proliferation and the NO can be calculated as a percentage of the untreated control.

15 Macrophage activation can also be assessed by expression of surface markers. Briefly,  $2 \times 10^6$  freshly isolated monocytes are divided in half and plated in a 12-well plate. One well can be activated with LPS and IFN- $\gamma$  as described above. After 24hr the cells in each well are harvested and divided into two tubes. One tube can be stained with CD11b/Mac1-PE (clone ICRF44), CD54/ICAM1-APC (clone HA58), CD80/B7.1-  
20 FITC (clone L307.4) (BD Pharmingen) and panMHCII-PerCP-Cy5.5 (Ancell, clone:TDR31.1) antibodies. The cells in the other tube are stained with isotype controls for compensation. These samples are then run through a FACScalibur instrument (Beckton Dickinson) and analyzed with Cellquest software (ver.3.1) as done previously. Activation can be determined as the difference in MFI between the LPS +  
25 IFN- $\gamma$  treated sample and the unstimulated control.

***(g) DCP Frequency and Dendritic Cell  
Differentiation.***

343. The DCP frequency assays are performed together with the OCP frequency assays immediately after monocyte purification by four-color FACS as  
30 described above. The DCP frequency can be presented as the % of CD11c stained cells that have a MFI greater than 99% of the APC-conjugated isotype control stained cells. Dendritic cell differentiation assays are performed as described in Figure 28.

Briefly,  $2 \times 10^6$  monocytes are incubated in 2 ml of RPMI-10% FCS medium supplemented with 100 ng/ml GM-CSF and 100 ng/ml IL-4 (R&D Systems) for 6 days. On days 2 and 4, half of the supernatant can be replaced with fresh media containing GM-CSF and IL-4. Afterwards the cells are harvested and divided into two tubes. The cells in the first tube are stained with CD209-PE (E Bioscience clone h209), CD1a-APC (clone HI149), CD64-FITC (clone 10.1) and CD14-percp Cy5.5 (clone M $\phi$ P9) (BD Pharmingen). The cells in the other tube are stained with isotype controls. These samples are then analyzed by FACS as described above. Dendritic cell differentiation can be quantified by determining the % of CD1a stained cells that have a MFI greater than 99% of the APC-conjugated isotype control stained cells. It can also be quantified as the % conversion of CD11b DCP to CD1a mature dendritic cells. Results with CD209 are used as a positive control for the IL-4 treatment and CD14 and CD64 are used to identify the macrophage population.

***(h) Serum ELISA.***

344. ELISA to determine the serum TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 levels can be performed as described previously. Briefly, 10ml of blood from each individual can be collected in a serum separation tube (Beckton Dickenson), and centrifuged for 10min at 2,500 RPM. The serum can be decanted and aliquoted into 1ml cryovials (Falcon) and stored at -80°C. Once all of the serum samples from all of the groups have been collected the concentration of TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 in the samples can be determined using specific sandwich ELISA (Amgen, R&D Systems or Endogen) according to the manufactures instructions. The data are presented as ng/ml of serum.

***(i) Data Analysis and Interpretation:***

345. Statistical analyses for each outcome measure can be performed by calculating the mean  $\pm$ SEM of the group (normal, PsA, nonerosive SLE, erosive SLE) and performing a multiparameter analysis of variance test (ANOVA). The results of the patient groups compared to the normal healthy controls as outlined in the table below.



	<u>PsA</u>	<u>PsA+anti-TNF</u>	<u>nonerosive SLE</u>	<u>erosive SLE</u>
OCP frequency	higher	normal	lower	
DCP frequency	lower	normal	higher	
CFU-M	higher	normal	lower	lower
M $\phi$ activation	lower	normal	lower	lower
TNF, M-CSF, RANKL	higher	normal	normal	
IFN, GM-CSF, IL-4	normal	normal	higher	higher

**f) Demonstrate the direct role of systemic TNF, IFN, and the combination of these cytokines on monocyte differentiation and erosive arthritis in mice.**

346. Introduction of a systemic TNF signal biases monocyte differentiation towards osteoclastogenesis and away from the macrophage and dendritic cell lineages resulting in erosive inflammatory arthritis in mice. Introduction of a systemic IFN signal biases monocyte differentiation towards dendritic cell differentiation and away from the macrophage and osteoclast lineages resulting in a nonerosive phenotype. In the presence of both signals, IFN dominates over TNF such that monocyte differentiation can be biased towards dendritic cell differentiation and away from the macrophage and osteoclast lineages resulting in a nonerosive phenotype. Thus, the TNF/IFN axis in inflammatory arthritis dictates erosive disease via these mechanisms.

347. Findings show that systemic TNF induces the release of large numbers of osteoclast precursors from the bone marrow into the blood, which must home to sites of inflammation in the bone microenvironment and receive a RANKL signal to differentiate into active osteoclasts, in order to mediate periarticular erosions. Simultaneously, work in the NZB x NZW mouse model of lupus has demonstrated the presence and critical role of systemic IFN in disease pathogenesis. The dominant role of IFN signaling in OCP resulting in the inhibition of inflammatory bone loss and maintenance of bone homeostasis has also been demonstrated in *Ifnr1*<sup>-/-</sup> mice (Takayanagi, H., et al. 2002. *Nature* 416:744). Having generated the TNF-Tg, *Nba2* and *Ifnr1*<sup>-/-</sup> mice in a pure C57B/6 background, it can be shown that the TNF/IFN axis in monocyte differentiation dictates the erosive phenotype in inflammatory arthritis using gain and loss of function studies.

**(1) Experimental Design and Methods:**

348. The gain-of-function effects of introducing systemic TNF, IFN and their combination on OCP and DC and macrophage activation in PBMC and splenocytes of C57B/6 mice can be determined and examine the impact this has on erosive arthritis can be shown by quantitative histomorphometry. In these experiments it is understood that one can use control C57B/6, C57B/6 injected with TNF, C57B/6 injected with poly I:C, TNF-Tg, TNF-Tg injected with poly I:C, (*Nba2* x NZW)F1, (*Nba2* x NZW)F1

injected with TNF, and (TNF-Tg x Nba2 x NZW)F1. Furthermore, serum TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 levels can be determined by ELISA.

349. Additionally, the loss-of-function effects of removing systemic TNF, IFN and their combination on OCP and DC and macrophage activation in PBMC and splenocytes of C57B/6 mice can be determined and the impact this has on erosive arthritis by quantitative histomorphometry examined. These experiments can utilize control C57B/6, TNF-Tg treated with anti-TNF, and (TNF-Tg x Nba2 x NZW x IFNAR1<sup>-/-</sup>). Furthermore, serum TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 levels by ELISA can be determined.

***(a) Genotyping and generation of congenic mice.***

350. C57B/6 and TNF-Tg mice in a pure C57B/6 background are propagated by mating C57B/6 females to TNF-Tg heterozygous males, to produce both WT controls and TNF-Tg mice with normal Mendelian distribution. 21 days after birth, the mice are ear punched and a tail biopsy can be taken to determine the genotype by PCR as described. Similarly, Nba2 locus has been identified in the NZB mice and crossed into a pure C57B/6 background (Rozzo, S. J., et al. 2001. *Immunity* 15:435). These mice (Nba2) can be mated with NZW/LacJ mice from Jackson Laboratories (NZW) to generate the F1 hybrids that develop lupus. To evaluate lupus in these mice in a type I IFN null background, the Nba2 mice and the NZW mice were crossed with the Ifnr1<sup>-/-</sup> mice. Matings with the TNF-Tg mice generates the TNF-Tg x Nba2 line and the TNF-Tg x Nba2 x Ifnr1<sup>-/-</sup> line. The genotyping for the human TNF transgene and the Ifnr1 null mutation are done by PCR as done previously (Schwarz, E. M., et al. 1998. *J. Virol.* 72:5654). Genotyping can be done for the Nba2 locus by assaying for the polymorphic (between NZB and B6) microsatellite markers D1Mit36, D1Mit113, Crp, D1Mit541, and D1Mit166, as described previously (Rozzo, S. J., et al. 2001. *Immunity* 15:435). This ensures that the full interval is inherited. These mice can be crossed to the NZW and NZW x Ifnr1<sup>-/-</sup> mice to generate the TNF-Tg x (Nba2 x NZW)F1 and TNF-Tg x Nba2 x NZW x Ifnr1<sup>-/-</sup> mice. In all cases, age matched, female mice can be used for the experiments.

***(b) TNF, anti-TNF and poly I:C administrations.***

351. Acute administration of 10µg murine TNFα (Amgen) can be injected i.p. daily as described previously. The murine anti-TNF-α mAb (Centocor) used in this study binds with high affinity to hTNFα (Knight, D. M., et al. 1993. *Mol Immunol* 30:1443) and effectively cures arthritis in the hTNF-Tg mouse (Keffer, J., et al. 1991. *Embo J* 10:4025). For acute administration, 200µg anti-TNF-α mAb can be administered once i.p. For chronic administration 200µg anti-TNF-α mAb can be administered weekly i.p. as described previously (Shealy, D. J., et al. 2002. *Arthritis Res* 4). For acute poly (I:C) (Sigma) administration, 100µg can be injected daily i.p. as described previously.

**(c) Experimental Groups.**

352. The most efficient and rigorous approach is to independently evaluate the effects of the TNF/IFN axis on monocyte differentiation acutely and chronically. The acute experiments are designed to provide information on direct effects, but are too short to evaluate effects on the erosive phenotype. The chronic experiments are design to provide information on the long-term effects of dysregulated TNF/IFN signaling on monocyte differentiation and the pathogenesis of erosions in inflammatory arthritis. An outline of the experimental groups and the outcome is listed below.

**Acute gain of TNF and IFN on osteoclastogenesis, macrophage activation and dendritic cell differentiation.**

Seven groups (N=10) of 3 month-old mice are treated as follows and sacrificed on day 3:

**Phenotype**

Group 1 wild-type C57B/6 placebo (100µl PBS i.p.) every day for 3 days  
**normal**

Group 2 wild-type C57B/6 TNF (10µg of TNF in 100µl i.p.) every day for 3 days  
**OCP high**

Group 3 wild-type C57B/6 IFN (100µg of poly I:C in 100µl i.p.) every day for 3 days  
**DCP high**

Group 4 TNF-Tg placebo (100µl PBS i.p.) every day for 3 days  
**OCP high**

Group 5 TNF-Tg IFN (100µg of poly I:C in 100µl i.p.) every day for 3 days  
**DCP high**

Group 6 (Nba2 x NZW)F1 placebo (100µl PBS i.p.) every day for 3 days  
**DCP high**

Group 7 (Nba2 x NZW)F1 TNF (10µg of TNF in 100µl i.p.) every day for 3 days  
**DCP high**

**Acute loss of TNF and IFN on osteoclastogenesis, macrophage activation and dendritic cell differentiation.**

Seven groups (N=10) of 3 month-old mice are treated as follows and sacrificed on day

**3: Phenotype**

Group 1 wild-type C57B/6 placebo (100µl PBS i.p.) once	
<b>normal</b>	
Group 2 wild-type C57B/6 anti-TNF (200µg of anti-TNF in 100µl i.p.) once	
<b>normal</b>	
Group 3 TNF-Tg placebo (100µl PBS i.p.) once	
<b>OCP high</b>	
Group 4 TNF-Tg anti-TNF (200µg anti-TNF in 100µl i.p.) once	
<b>normal</b>	
Group 5 (Nba2 x NZW)F1 placebo (100µl PBS i.p.) once	<b>DCP high</b>

**Chronic gain of TNF and IFN on osteoclastogenesis, macrophage activation, dendritic cell differentiation and erosive arthritis.****Four strains of mice are analyzed at 3, 6, 9 and 12 months of age (N=10): PBMC & bone phenotype**

Group 1 wild-type C57B/6	<b>normal-normal</b>
<b>normal</b>	
Group 2 TNF-Tg	<b>OCP high-erosive</b>
<b>erosive</b>	
Group 3 (Nba2 x NZW)F1	<b>DCP high-normal</b>
<b>normal</b>	
Group 4 TNF-Tg x (Nba2 x NZW)F1	<b>DCP high-nonerosive</b>

**Chronic loss of TNF and IFN on osteoclastogenesis, macrophage activation, dendritic cell differentiation and erosive arthritis.****Four strains of mice are analyzed at 3, 6, 9 and 12 months of age (N=10): PBMC & bone phenotype**

Group 1 wild-type C57B/6 placebo (100µl PBS i.p.) weekly	<b>normal-normal</b>
Group 2 wild-type C57B/6 anti-TNF (200µg in 100µl i.p.) weekly	<b>normal-normal</b>
Group 3 Ifnr1-/-	<b>OCP high-osteoporotic</b>
<b>osteoporotic</b>	
Group 4 TNF-Tg placebo (100µl PBS i.p.) weekly	<b>OCP high-erosive</b>
Group 5 TNF-Tg anti-TNF (200µg in 100µl i.p.) weekly	<b>normal-normal</b>
<b>normal</b>	
Group 6 (Nba2 x NZW)F1	<b>DCP high-normal</b>
Group 7 (Nba2 x NZW)F1 x Ifnr1-/-	<b>normal-osteoporotic</b>
<b>osteoporotic</b>	
Group 8 TNF-Tg x (Nba2 x NZW)F1 placebo (100µl PBS i.p.) weekly	<b>DCP high-nonerosive</b>
Group 9 TNF-Tg x (Nba2 x NZW)F1 anti-TNF (200µg in 100µl i.p.) weekly	<b>DCP high-normal</b>
<b>high-normal</b>	
Group 10 TNF-Tg x (Nba2 x NZW)F1 x Ifnr1-/- placebo (100µl PBS i.p.) weekly	<b>OCP high-erosive/osteoporotic</b>
<b>OCP high-erosive/osteoporotic</b>	
Group 11 TNF-Tg x (Nba2 x NZW)F1 x Ifnr1-/- anti-TNF (200µg in 100µl i.p.) weekly	<b>OCP-osteoporotic</b>

***(d) Collection of serum, PBMC and splenocytes.***

353. At the time of sacrifice, the mice are euthanized by cervical dislocation and the chest cavity can be opened to collect blood via ventricular nicking. This method produces 1.5-2.0ml of blood per mouse. The first 200ml of blood are used to obtain serum after clotting and stored at  $-80^{\circ}\text{C}$  for ELISA and future studies as described previously. The rest of the blood can be washed in PBS and resuspended in red cell lysis buffer and monocytes are isolated from a ficol gradient as described previously. This method produces >1million cells which can be sufficient to determine OCP frequency by FACS and TRAP assay as described in Figure 22. Splenocytes are prepared from a tissue squash followed by red cell lysis and ficol gradient purification as described previously. This method produces >10million cells which can be sufficient to determine OCP frequency, DC activation, CFU-M colonies, and macrophage activation.

***(e) OCP Frequency and Osteoclastogenesis.***

354. OCP and DCP frequency can be determined simultaneously, immediately after monocyte purification of PBMC and splenocytes, by four-color FACS exactly as described in the human study above. The murine specific antibodies to be used are CD11b-PE (clone M1/70), CD11c-APC (clone HL3), CD80-FITC (16-10A1) and CD8-PerCP-Cy5.5 (clone 53-6.7) (BD Pharmingen). The OCP frequency can be determined by the % of CD11b<sup>hi</sup> cells as described previously. TRAP osteoclastogenesis can be performed as described previously.

***(f) CFU-M Colony Formation and Macrophage Activation.***

355. The *in vitro* colony-forming assay can be performed as above, with splenocytes as described previously. Macrophage activation can be determined by NO respiratory burst assays and FACS analysis of splenocytes as described above. The murine specific antibodies used are CD11b/Mac1-PE (clone M1/70), CD54/ICAM1-APC (clone 3E2), CD80/B7.1-FITC (clone 16-10A1) (BD Pharmingen) and I-A<sup>b</sup>-PerCP-Cy5.5 (clone AF6-120.1) antibodies. Activation can be determined as the difference in MFI between the LPS + IFN- $\gamma$  treated sample and the unstimulated control.

***(g) DCP Frequency and Dendritic Cell Activation.***

356. The DCP frequency assays are performed together with the OCP frequency assays immediately after monocyte purification of PBMC and splenocytes by four-color FACS as described above. The splenocyte-derived DC activation in response to IFN- $\alpha$  are performed as previously described. Briefly,  $2 \times 10^6$  cells are incubated in 5 ml RPMI-10% FCS medium supplemented with 3 ng/ml GM-CSF and 5 ng/ml IL-4 (BD Biosciences) for 6 days. On days 3 and 6, half of the supernatant can be replaced with fresh media containing GM-CSF and IL-4. To induce final maturation, 1,000 U/ml IFN- $\alpha$  (Calbiochem) can be added for another 24 h. Control cells receive media alone. The cells are then analyzed with murine specific antibodies against CD11c, CD80, I-A<sup>b</sup>, and H-2K<sup>b</sup> by FACS as described above. Activation can be determined as the difference in MFI between the IFN- $\alpha$  treated sample and the unstimulated control.

***(h) Serum ELISA.***

357. ELISA to determine the serum TNF $\alpha$ , IFN $\alpha$ , M-CSF, RANKL, GM-CSF and IL-4 levels can be performed as described previously, using specific sandwich ELISA (Amgen, R&D Systems or Endogen) according to the manufactures instructions. The data are presented as ng/ml of serum.

358. The development of lupus in the mice can be monitored by serum Igs and autoantibodies (anti-dsDNA and anti-ssDNA) by ELISA, as described previously. Microtiter plates are coated with either 5  $\mu$ g/ml Fc-specific F(ab')<sub>2</sub> goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), 5  $\mu$ g/ml anti-mouse IgM (Southern Biotechnology Associates, Inc.), 25  $\mu$ g/ml calf thymus dsDNA, or 25  $\mu$ g/ml ssDNA. Total bound IgM or IgG, and IgG subclasses are measured by alkaline phosphatase-labeled goat anti-mouse Abs (Caltag Laboratories) and compared with a standard serum (Bethyl Laboratories).

***(i) Histomorphometry.***

359. The primary outcome measure to determine the erosive phenotype can be histomorphometry of the knee joints as described in Figure 25, specifically, the percent eroded surface (eroded surface/total joint surface in mm). Osteoclast numbers and bone volume are quantified to assess osteoporosis. This can be done by harvesting

the left knees at the time of sacrifice followed by formalin fixation. The tissues are then decalcified in EDTA, paraffin embedded and serial 3µm sections are stained with H&E and for TRAP as described previously. The mean of 3 TRAP stained sections 100µm apart represents the value per animal and used to derive the mean + SEM of the group (N=10). Statistically significant differences between groups can be determined by analysis of variance for multiple group comparisons (ANOVA), where  $p < 0.05$  can be considered to be significant.

360. As a secondary outcome measure to evaluate the arthritis, the H&E sections can be evaluated in a blinded manner by two independent observers (ES&CR) for histologic signs of arthritis and scored as described previously (Yin, G., et al. 2002. *Mol Ther* 5:547), where: 1, synovial cell proliferation, synovial hypertrophy with villus formation and/or fibrin deposition; 2, inflammation, synovitis and/or generalized inflammation; 3, cartilage disruption, chondrocyte degeneration and/or ruffling of cartilage surface and/or dystrophic cartilage; and 4, joint destruction, cartilage erosion with abundant inflammation and pannus formation with bone erosion. Non-parametric statistical analyses can be performed to determine statistical significance between groups in which  $p < 0.05$ .

361. The kidneys of the mice are also harvested at the time of sacrifice. They can be fixed in paraformaldehyde, rehydrated in sucrose and frozen in OTC block for storage at  $-80^{\circ}\text{C}$  as done previously (Schwarz, E. M., et al. 1997. *Genes Dev* 11:187). These tissues can be used to assess for evidence of lupus nephritis as described previously (Rozzo, S. J., et al. 2001. *Immunity* 15:435).

**(j) Radiographic evaluation.**

362. As an additional secondary outcome measure, erosions are evaluated radiographically with x-rays obtained in an anteroposterior position as described previously (Zhang, X., et al. 2002. *J Clin Invest* 109:1405). The radiographs are evaluated in a blinded manner by two independent observers (ES&CR) according to the Larsen method and statistical analyses can be performed accordingly (Larsen, A., and J. Thoen. 1987. *Scand J Rheumatol* 16:395).



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**H. Sequences**

1. SEQ ID NO:1 RANKL anti-sense 5'
2. SEQ ID NO:2 RANK sense 5'
3. SEQ ID NO:3 RANK anti-sense 5'
4. SEQ ID NO:4 OPG sense 5'
5. SEQ ID NO:5 OPG anti-sense 5'
6. SEQ ID NO:6 GAPDH sense 5'
7. SEQ ID NO:7 GAPDH anti-sense 5'
8. SEQ ID NO:8
9. SEQ ID NO:9 RANKL AF019048 *Mus musculus* mRNA, complete cds
10. SEQ ID NO:10 RANKL AF019048 *Mus musculus* Protein
11. SEQ ID NO:11 RANKL AF019047 *Mus musculus* mRNA, complete cds
12. SEQ ID NO:12 RNKL AF019047 *Homo Sapiens* Protein
13. SEQ ID NO:13 RANK AF018253 *Homo sapiens* receptor activator of nuclear factor-kappa B (RANK) mRNA
14. SEQ ID NO:14 RANK AF018253 *Homo sapiens* receptor activator of nuclear factor-kappa B (RANK) Protein
15. SEQ ID NO:15 RANK AF019046 *Mus musculus* receptor activator of nuclear factor-kappa B (RANK) mRNA
16. SEQ ID NO:16 RANK AF019046 *Mus musculus* receptor activator of nuclear factor-kappa B (RANK) Protein
17. SEQ ID NO:17 TNF alpha homo sapiens mRNA
18. SEQ ID NO:18 TNF alpha homo sapiens protein
19. SEQ ID NO:19 TNF alpha NM\_013693 *Mus musculus* mRNA
20. SEQ ID NO:20 TNF alpha NM\_013693 *Mus musculus* mRNA
21. SEQ ID NO:21 TNFR1 M75866 Human tumor necrosis factor receptor 1 (TNFR1) complete cds

- 22. SEQ ID NO:22 TNFR1 M75866 Human tumor necrosis factor receptor 1 (TNFR1) Protein**
- 23. SEQ ID NO:23 TNFR1 L26349 Mus Musculus tumor necrosis factor receptor 1 (TNFR1) complete cds**
- 24. SEQ ID NO:24 TNFR1 L26349 Mus Musculus tumor necrosis factor receptor 1 (TNFR1) Protein**
- 25. SEQ ID NO. 25 CD11b primers**
- 26. SEQ ID NO. 26 CD11b primers**
- 27. SEQ ID NO. 27 actin primers**
- 28. SEQ ID NO. 28 actin primers**